

# Effects of Naringenin on Glucose Uptake in L6 skeletal muscle cells

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## ABSTRACT

Diabetes mellitus is a disorder of inadequate insulin action and consequent high blood glucose levels. Type 2 diabetes accounts for the majority of cases of the disease and is characterized by insulin resistance and relative insulin deficiency resulting in metabolic deregulation. It is a complex disorder to treat as its pathogenesis is not fully understood and involves a variety of defects including  $\beta$ -cell failure, insulin resistance in the classic target tissues (adipose, muscle, liver), as well as defects in  $\alpha$ -cells and kidney, brain, and gastrointestinal tissue. Present oral treatments, which aim at mimicking the effects of insulin, remain limited in their efficacy and therefore the study of the effects of novel compounds on insulin target tissues is an important area of research both for potentially finding more treatment options as well as for increasing our knowledge of metabolic regulation in health and disease.

In recent years the extensively studied polyphenol, resveratrol, has been reported to have antidiabetic effects showing that it increases glucose uptake by skeletal muscle cells and prevents fatty acid-induced insulin resistance *in vitro* and *in vivo*. Naringenin, a citrus flavonoid with structural similarities to resveratrol, is reported to have antioxidant, antiproliferative, anticancer, and anti-inflammatory properties. Effects on glucose and lipid metabolism have also been reported including blood glucose and lipid lowering effects. However, whether naringenin has insulin-like effects is not clear. In the present study the effects of naringenin on glucose uptake in skeletal muscle cells are examined and compared with those of insulin. Naringenin treatment of L6 myotubes increased glucose uptake in a dose- and time-

dependent manner and independent of insulin. The effects of naringenin on glucose uptake achieved similar levels as seen with maximum insulin stimulation and its effect was additive with sub-maximal insulin treatment. Like insulin naringenin treatment did not increase glucose uptake in myoblasts. To elucidate the mechanism involved in naringenin action we looked at its effect on phosphatidylinositol 3-kinase (PI3K) and Akt, two signalling molecules that are involved in the insulin signalling cascade leading to glucose uptake. Naringenin did not stimulate basal or insulin-stimulated Akt phosphorylation but inhibition of PI3K by wortmannin partially repressed the naringenin-induced glucose uptake. We also examined naringenin's effect on AMP-activated protein kinase (AMPK), a molecule that is involved in mediating glucose uptake by a variety of stimuli. Naringenin stimulated AMPK phosphorylation and this effect was not inhibited by wortmannin. To deduce the nature of the naringenin-stimulated AMPK phosphorylation and its impact on glucose uptake we examined the role of several molecules implicated in modulating AMPK activity including SIRT1, LKB1, and  $ca^{2+}$ /calmodulin-dependent protein kinase kinase (CaMKK).

Our results indicate that inhibition of SIRT1 did not prevent the naringenin-stimulated glucose uptake or AMPK phosphorylation; naringenin did not stimulate LKB1 phosphorylation; and inhibition of CaMKK did not prevent naringenin-stimulated glucose uptake. Inhibition of AMPK by compound C also did not prevent naringenin-stimulated glucose uptake but effectively inhibited the phosphorylation of AMPK suggesting that AMPK may not be required for the naringenin-stimulated glucose uptake.

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## List of Abbreviations

**2DG:** 2-[<sup>3</sup>H]deoxy-D-glucose  
**α-MEM:** Minimum essential medium  
**ACAT:** Acyl-CoA:cholesterol acyltransferase  
**ADP:** Adenosine diphosphate  
**AICAR:** 5-aminoimidazole-4-carboxamide ribonucleoside; a nucleoside that is converted when take up by cells into an AMP analogue, ZMP  
**AMP:** Adenosine monophosphate  
**AMPK:** AMP-activated protein kinase  
**ApoB:** Apolipoprotein B  
**Apo-Lp:** ApoB-containing lipoproteins  
**AS160:** Akt substrate 160kDa  
**ATP:** Adenosine triphosphate  
**BBMV:** Brush border membrane vesicles  
**CaMKK:** Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase  
**CB:** Cytochalasin B  
**CBS:** Cystathionine-β-synthase  
**CC:** Compound C; an AMPK inhibitor  
**CE:** Cholesteryl ester  
**CHO:** Chinese hamster ovary  
**CR:** Caloric restriction  
**DD:** Distilled water  
**DGAT:** Diacylglycerol acyltransferase  
**DN:** Dominant-negative  
**ER:** Endoplasmic reticulum or estrogen receptor depending on context  
**ERK:** Extracellular signal-regulated kinase  
**FBS:** Fetal bovine serum  
**GLUTs:** A family of sodium-independent glucose transporters  
**Grb-2:** Growth factor receptor binding protein 2  
**HBS:** HEPES Buffer Saline  
**HMG-CoA:** 3-hydroxy-3-methylglutaryl-coenzyme A  
**HRP-conjugated:** Horseradish peroxidase  
**IGF:** Insulin-like growth factor  
**ILK:** Integrin-linked kinase  
**IR:** Insulin resistance or insulin receptor depending on context  
**IRR:** Insulin receptor-related receptor  
**IRS:** Insulin receptor substrate  
**K<sub>m</sub>:** Michaelis constant  
**L6:** Refers to the L6 skeletal muscle cell line  
**MCP-1:** Monocyte chemotactic protein-1  
**MO25:** Mouse protein 25  
**mTOR:** Mammalian target of rapamycin  
**MTP:** Microsomal triglyceride transfer protein  
**NAD:** Nicotinamide adenine dinucleotide  
**PBS:** Phosphate buffer saline

**PDGF:** Platelet-derived growth factor  
**PDK:** Phosphoinositide-dependent kinase  
**PH:** Pleckstrin homology  
**PI3K:** Phosphatidylinositol 3-kinase or phosphoinositide 3-kinases  
**PIP<sub>3</sub>:** Phosphatidylinositol-3-phosphates  
**PMSF:** Serine protease inhibitor  
**PKB:** Protein kinase B; also known as Akt  
**PKC:** Protein kinase C  
**PLD:** Phospholipase D  
**PVDF:** Polyvinylidene difluoride  
**PYK-2:** Proline-rich tyrosine kinase-2  
**S6K1:** S6 kinase 1  
**SDS:** Sodium dodecyl sulphate  
**SDS-PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
**SE:** Standard error  
**Ser:** Serine  
**SH2:** Src-homology-2  
**SHP-2:** SH2-containing protein-tyrosine phosphatase-2  
**SGLT:** Sodium-dependent glucose transporter  
**siRNA:** Small interference RNA  
**SoHo:** Sorbin homology  
**SREBP:** Sterol regulatory element-binding protein  
**STO:** STO-609  
**STRAD:** STE20-related adaptor protein  
**STZ:** Streptozotocin  
**TBS:** Tris-buffered saline  
**TG:** triglycerides  
**Thr:** Threonine  
**TSC:** Tuberous Sclerosis Complex  
**VCAM-1:** Vascular cell adhesion molecule-1  
**VLDL:** Very low-density lipoprotein  
**V<sub>max</sub>:** Maximum rate  
**ZMP:** 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranosyl-5'-monophosphate; an AMP analogue

## CHAPTER 1: INTRODUCTION

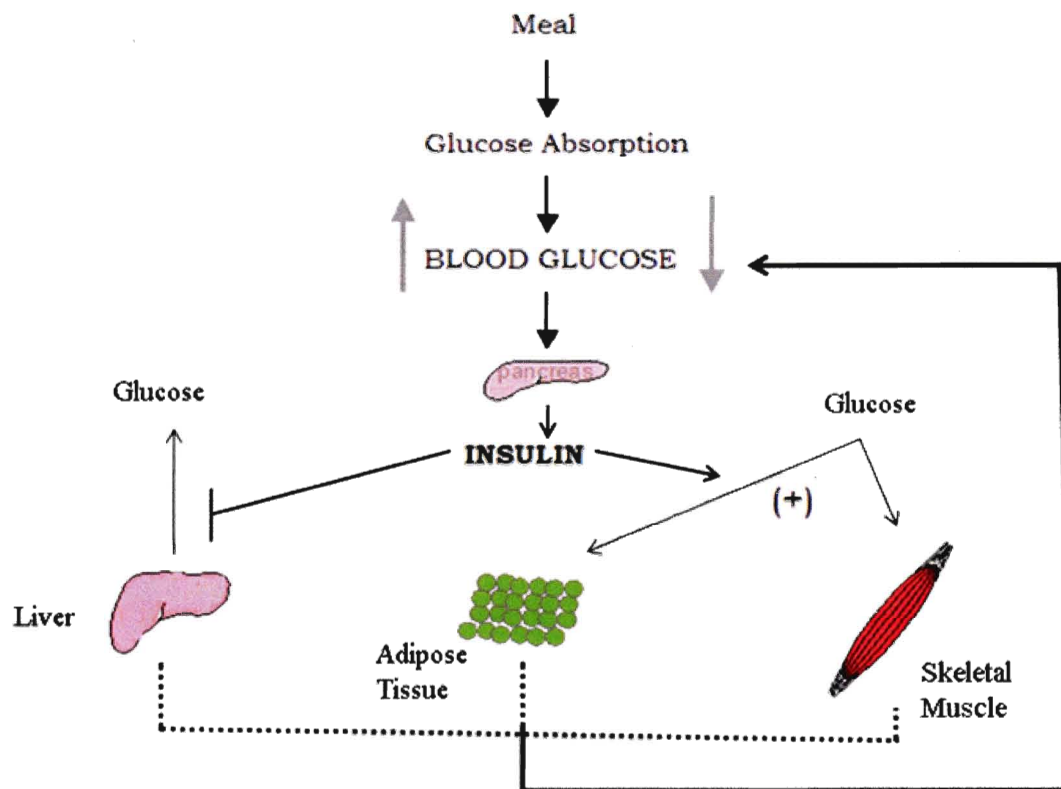
### 1.1 Insulin

Insulin is a protein hormone produced by the  $\beta$  cells of the endocrine portion of the pancreas, the islets of Langerhans. Upon binding to its receptor on target cells insulin activates signalling cascades that lead to diverse biological effects including glucose and lipid uptake and metabolism, gene expression, protein synthesis, and cell growth, division and survival (55,99). Of its many roles insulin is particularly important in maintaining glucose homeostasis. When the blood glucose level becomes elevated the change is detected by the pancreas, which responds by releasing insulin into the blood stream. Insulin helps to bring high blood glucose levels back to normal by inhibiting liver glycogenolysis and gluconeogenesis, by stimulating entry of glucose into tissue targets such as skeletal muscle, adipose and cardiac muscle, and promoting glycogen synthesis (Figure 1) (99,102).

Glucose is a crucial source of energy for the body and due to being a vital source for some tissues the body favours the utilization of other fuels, and storage of glucose unless stores are high in order to maintain consistent blood glucose levels. It is therefore important to have an effective system regulating blood glucose. Insulin is critical in this process as it facilitates the transport of glucose into target cells for utilization and storage when blood glucose levels increase. Glucose is a polar carbohydrate with five hydroxyl groups and it cannot pass through the hydrophobic plasma membranes of cells. With some exceptions it is transported into cells through facilitated diffusion by specialized glucose transporter proteins called GLUTs that open a gateway for glucose entry. There are 14 different GLUT isoforms the most

studied of which are GLUTs 1-4, and these transporters exhibit tissue specific expression. Although there are certain constitutive GLUTs present on the plasma membrane of all cells in the body, they cannot sufficiently maintain normoglycemia of the ever-changing environment. GLUT1 is one such transporter, which is largely responsible for glucose transport under basal conditions (55,102).

However, once blood glucose levels become elevated the glucose transporters available on plasma membranes are not sufficient to remove the glucose from the blood. Therefore the body requires more glucose transporters in order to increase the permeability of the plasma membrane to glucose and the success of achieving this largely depends on the availability of the GLUT4 transporters in cells that express the GLUT4 transport gene. The availability of GLUT4 transporters at the plasma membrane, unlike that of other glucose transporters, is mainly regulated by insulin. Glucose transport via this system is primarily confined to muscle and adipose cells (55,99). In cardiac muscle, skeletal muscle and adipose tissue the insulin signal leads to the translocation of GLUT4 proteins from intracellular stores to the plasma membrane thereby increasing glucose transport into the tissues (4.1,112). GLUT4-containing vesicles continuously cycle from intracellular stores to the plasma membrane but insulin stimulation increases the rate of GLUT4-vesicle exocytosis and reduces the rate of endocytosis thus leading to more GLUT4 transporters at the plasma membrane (56.1,102). Skeletal muscle accounts for the majority, ~80%, of the insulin-stimulated glucose uptake, which makes this tissue an important component of glucose homeostasis (53,85).



**Figure 1:** Role of insulin in maintaining blood glucose levels. An increase in blood glucose levels occurs after a meal which will stimulate the pancreas to release insulin into the circulation. Major insulin targets include the liver, adipose tissue, and skeletal muscle. Insulin inhibits glycogenolysis and gluconeogenesis in the liver, and stimulates muscle and adipose tissue glucose uptake and glycogen synthesis.

## 1.2 The Insulin Receptor

The insulin receptor is a plasma membrane intrinsic tyrosine kinase receptor and is part of a subfamily of receptor tyrosine kinases, which also includes the insulin-like growth factor (IGF)-I receptor and the insulin receptor-related receptor (IRR) (102). The receptor is composed of two monomers; an  $\alpha$  and a  $\beta$  subunit linked together by a disulfide bond. The two monomers are linked by two disulfide bridges between the two  $\alpha$  subunits (10). The  $\alpha$  subunits are entirely localized in the extracellular fluid and contain the insulin-binding domain while the  $\beta$  subunits start at the extracellular fluid, span the plasma membrane, and extend into the cytosolic surface where the tyrosine kinase is located. The intrinsic tyrosine kinase activity of the insulin receptor is essential for insulin action (99). The precise mechanism of insulin action on glucose uptake remains elusive but parts of the signalling pathway have been well established (Figure 2). In the unbound state the  $\alpha$  subunit inhibits the tyrosine kinase activity of the  $\beta$  subunit. Upon binding to the  $\alpha$  subunits of its receptor insulin removes this suppression of the kinase activity which is followed by autophosphorylation of the  $\beta$  subunits and conformational changes of the receptor leading to increased activity of the intrinsic tyrosine kinase domain of the receptor (102). The phosphorylated tyrosine residues of the insulin receptor recruit a variety of intracellular downstream target proteins. At least nine of these downstream targets have been identified of which four belong to a family of insulin receptor substrate (IRS) proteins while the remaining include Gab-1, p60<sup>dok</sup>, Cbl, APS, and isoforms of Shc (102).

### 1.3 IRS

The IRS family of proteins consists of at least four isoforms (IRS-1 to -4), which differ in their tissue distribution, cellular localization and function. The role of IRS-1 in metabolic regulation is mainly focused in skeletal muscle and adipose tissue and includes involvement in protein synthesis and glucose transport. The IRS-2 is expressed in liver, adipose, and muscle tissue where it has a broad range of activity, the IRS-3 is predominantly expressed in adipose tissue and the IRS-4 in neuroendocrine tissue. Although the elucidation of the respective role(s) of IRS-3 and IRS-4 proteins is not complete studies suggest that they may negatively regulate IRS-1 and -2 (99,102). Phosphorylated tyrosine residues of the IRS proteins act as docking sites for a range of downstream effector proteins that contain a Src-homology-2 (SH2) such as phosphatidylinositol 3-kinase (PI3K), growth factor receptor binding protein 2 (Grb-2), and SH2-containing protein-tyrosine phosphatase-2 (SHP-2) (99,102). The specific insulin response generated by a cell depends on which of these downstream effector molecules are expressed and which of them are recruited to the phosphorylated IRS protein (99).

### 1.4 PI3K

PI3K is composed of a p110 catalytic subunit and a p85 regulatory subunit that has two SH2 domains which interact with specific phosphotyrosine-containing motifs in the IRS proteins, pYMXM and pYXXM (5,102). There are eight isoforms of p85 regulatory subunit identified which are derived from three genes. The isoform that is believed to be responsible for mediating most responses is p85 $\alpha$  (102). PI3K is activated when the SH2 domain of its p85-regulatory subunit binds to the

phosphorylated tyrosine residues located on substrates such as IRS-1. Upon binding PI3K becomes active and it catalyses the phosphorylation of 3'-phosphoinositides to produce phosphatidylinositol-3-phosphates, particularly (PI3,4,5P<sub>3</sub>). Phosphatidylinositol-3-phosphate binds to the pleckstrin homology (PH) domains of various molecules resulting in alteration of their activity or cellular localization (102). Aside from its function in the production of phosphatidylinositol-3-phosphates, PI3K also has serine kinase activity and the catalytic and regulatory subunits are able to interact with other proteins. There are three main classes of molecules regulated by phosphatidylinositol-3-phosphates: the AGC family of serine/threonine protein kinases, guanine nucleotide-exchange proteins of the Rho family of GTPases, and the TEC family of tyrosine kinases (102). One kinase of the AGC family is phosphoinositide-dependent kinase 1 (PDK1). Although the precise interaction remains to be determined 3'-phosphoinositides affect two serine/threonine kinases, PDK1 and Akt (also known as PKB) (114). PI3K has been confirmed to play a role in metabolic signalling, and in skeletal muscle cells it has been established that stimulation of the PI3K pathway by insulin is crucial for the insulin-stimulated glucose uptake. Inhibition of PI3K activity has been shown to completely abolish glucose uptake and GLUT4 translocation in skeletal muscle and adipose cells (15.1,55).

### **1.5 Akt**

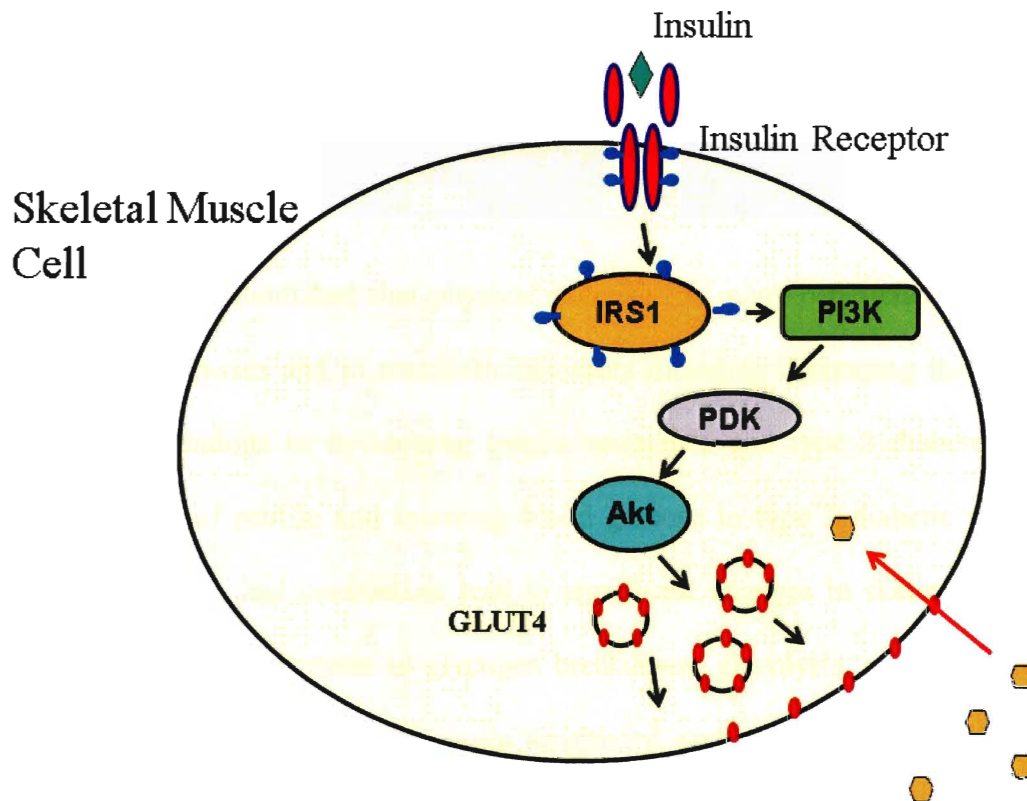
Akt is a serine/threonine kinase with three isoforms identified in mammals; Akt1, Akt2, and Akt3. Full activation of Akt requires phosphorylation of two of its residues, threonine residue (Thr308) and serine residue (Ser473). The mechanism of



this phosphorylation is not completely understood. It still remains unclear how the serine residue becomes phosphorylated, although the integrin-linked kinase (ILK) appears to be dependent on PI3K and is capable of phosphorylating the Ser473 site. It is known that the threonine residue is phosphorylated by PDK1 and this phosphorylation of Akt is thought to occur after polyphosphoinositide binding which increases Thr308 accessibility (112). It has been demonstrated that the Akt2 isoform is important in insulin-stimulated glucose uptake and GLUT4 translocation and it has been found to specifically associate with GLUT4 containing vesicles and to phosphorylate proteins associated with GLUT4 vesicle. The crucial downstream targets of Akt in glucose uptake remain to be identified (3,54,55). Substrates of Akt include the serine/threonine kinase GSK3 $\beta$ , proapoptotic proteins BAD and caspase 9, 6-phosphofructose-2-kinase, the forkhead family of transcription factors, and the Akt substrate 160kDa (AS160) (112,113). AS160 has been shown to mediate GLUT4 trafficking in L6 GLUT4 $myc$  expressing myoblasts. When AS160 is in its nonphosphorylated state it inhibits GLUT4 translocation to the plasma membrane, and phosphorylation of AS160 by Akt leads to GLUT4 translocation in skeletal muscle cells (113).

Although it has been well established that the activation of the IRS/PI3K/Akt signalling pathway is essential for insulin-stimulated glucose uptake and GLUT4 translocation, there is room to believe that other signalling molecules or pathways are required. So far no studies have been able to explain the specificity of the PI3K response to insulin. PI3K is activated by a variety of hormones and growth factors but only activation of PI3K by some of these activators results in glucose uptake and

GLUT4 translocation (31,48,112,113). And while in muscle both insulin and PDGF stimulate activation of PI3K and Akt phosphorylation, only insulin induces Akt phosphorylation in adipocytes (112,113). Additionally, studies in adipocytes using a cell permeable analog of PI3,4,5P<sub>3</sub> show that administration of the analog alone does not lead to glucose uptake while the co-administration of the analog with insulin into cells pre-treated with wortmannin, a very potent PI3K inhibitor, show enhanced glucose uptake. Further examination showed that the analog induced GLUT4 translocation but the transporters were not functional (112). This indicates that not only translocation but also activity of GLUT4 is important for the increase in glucose uptake. These experiments support the presence of a PI3K-independent pathway that is essential for glucose transport activation. As reviewed by Summers *et al* (112) other studies also demonstrate a lack of correlation between IRS-associated PI3K and insulin-stimulated glucose uptake and/or GLUT4 translocation, showing that although the insulin-stimulated IRS phosphorylation is required for some PI3K responses it is not required for GLUT4 translocation and that the association between IRS and PI3K may have little impact in glucose uptake even though PI3K is still required for GLUT4 translocation.



**Figure 2:** Key elements of the insulin signalling cascade leading to glucose uptake in skeletal muscle cells

### 1.6 The CAP/Cbl Pathway

The CAP/Cbl pathway seems to be another pathway required for insulin-stimulated glucose uptake. CAP belongs to a family of adaptor proteins that contain three SH3 domains and a sorbin homology (SoHo) domain. This protein associates with a Cbl molecule through its carboxyl-terminal SH3 domain. In this pathway the insulin receptor phosphorylates Cbl on tyrosine residues, which leads to the translocation of the Cbl-CAP complex to lipid raft domains in the plasma membrane. Once translocated the Cbl recruits the adaptor protein CrkII where CrkII forms a complex with the C3G protein. The movement of C3G, a guanyl nucleotide-exchanger, to the lipid rafts allows it to associate with the G-protein TC10 and catalyze

the exchange of GTP for GDP. This in turn activates TC10 which creates an additional signal for GLUT4 proteins (55,102).

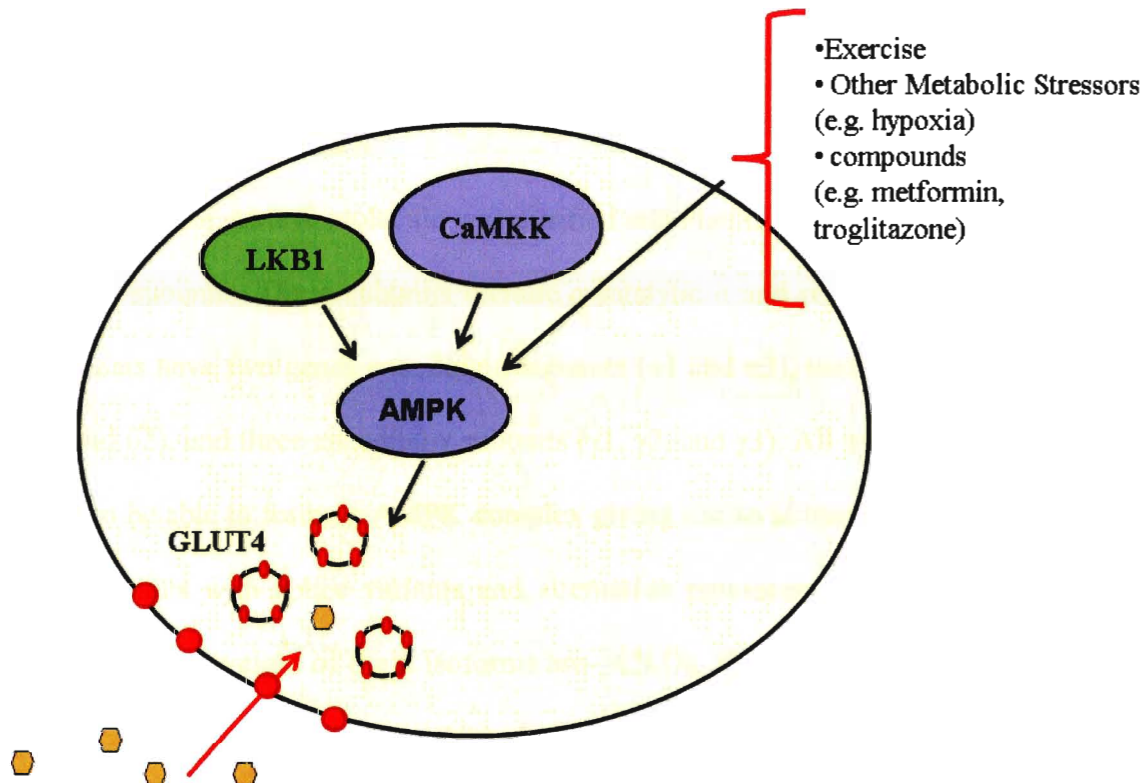
## **1.10 Other Mechanisms Mediating Glucose Uptake**

### **1.10.1 Exercise**

It has been established that physical exercise has many beneficial effects on physiological processes and in metabolic disorders including decreasing the risk of susceptible populations to developing insulin resistance and type 2 diabetes, and improving the lipid profile and lowering blood glucose in type 2 diabetic patients (28,51,86). Exercise and contraction lead to significant changes in skeletal muscle metabolism including increases in glycogen breakdown, glycolysis, glucose uptake, fatty acid oxidation, post-exercise insulin sensitivity, and leads to changes in gene expression (28,36). The mechanisms mediating the exercise-induced increase in glucose uptake have remained elusive. Evidence suggests that exercise stimulates glucose uptake in skeletal muscle is independent of the insulin signalling pathway. Acute exercise does not appear to stimulate the insulin signalling pathway, having no apparent effect on tyrosine phosphorylation of the insulin receptor or IRS-1, and does not increase PI3K activity. Furthermore the exercise-stimulated increase in glucose uptake is not impaired by inhibition of the insulin signalling pathway (83,101.1)

On the other hand there is strong evidence implicating AMP-activated protein kinase (AMPK) in regulating the short-term and long-term adaptations of exercise in skeletal muscle (28,84). Exercise and muscle contraction activates AMPK in rodent and human skeletal muscle, liver, and adipose cells (33,51,86,124). For instance treadmill exercise in rat skeletal muscle, sciatic nerve-stimulated muscle contractions

*in situ*, contraction of isolated muscle *in vitro*, and moderate to high exercise in humans all show increased AMPK activity (28,84,86,124). Thus various experimental models (treadmill exercise in rats, nerve-stimulated muscle contraction *in situ*, contraction of isolated muscle, exercise in humans) consistently show that exercise and contraction stimulate AMPK activity in muscle (84,86). Although there is extensive evidence showing that AMPK stimulates glucose uptake in skeletal muscle its mechanism of action and the signaling pathway that it is part of remains to be determined (Figure 3).



**Figure 3:** Other mechanism of increasing glucose uptake. AMPK is a sensor of cellular energy status and is stimulated by rising AMP to ATP ratio due to ATP depletion. Therefore it is activated by metabolic stresses that either inhibit ATP production (e.g. hypoxia) or increase ATP consumption (exercise, muscle contraction). Some antidiabetic drugs including metformin and troglitazone are reported to mediate their action via activation of AMPK.

### 1.10 AMPK

The genes that encode for AMPK are present in all eukaryotic cells for which genome sequences have been completed, from single-celled organisms such as budding yeast (*Saccharomyces cerevisiae*) to highly specialized mammals (35,37). In mammalian cells the AMPK system acts as a sensor of changes in cellular energy status, regulates numerous processes of cellular metabolism, integrates nutritional and

hormonal signals in the peripheral tissues and the hypothalamus, and mediates the effects of adipokines in regulating food intake, body weight and glucose and lipid homeostasis (13, 34, 51,56).

### 1.10.1 Structure of AMPK

In 1994 the AMPK molecule was purified and identified as a complex consisting of three subunits. These subunits include a catalytic  $\alpha$  and regulatory  $\beta$  and  $\gamma$  units. Mammals have two genes encoding  $\alpha$  subunits ( $\alpha 1$  and  $\alpha 2$ ), two encoding  $\beta$  subunits ( $\beta 1$  and  $\beta 2$ ), and three encoding  $\gamma$  subunits ( $\gamma 1$ ,  $\gamma 2$ , and  $\gamma 3$ ). All isoform combinations seem to be able to form an AMPK complex giving rise to at least 12 possible isoform combinations with splice variants and alternative promoters adding to the number. The molecular weight of the  $\alpha$  isoforms are  $\sim 63$ kDa, the  $\beta$  subunits are  $\sim 30$ kDa, and the  $\gamma$  subunits are variable ranging from  $\sim 37$ - $63$ kDa (13,28,118). The  $\alpha$  subunit contains a serine/threonine kinase catalytic domain located at the N-terminal half (13,35,). It also has several residues that can be phosphorylated (Thr172, Thr258, and Ser485 ( $\alpha 1$ )/491 ( $\alpha 2$ )) and the phosphorylation by upstream kinases of the Thr172 residue located on the activation loop of the kinase's catalytic domain is necessary for activation of AMPK. Site directed mutation of Thr172 to alanine renders the kinase inactivity, and phosphorylation of Thr172 mimics the activity of AMPK under all tested conditions (13,35). The C-terminus of the  $\alpha$  subunit contains a sequence required for formation of the complex with the other subunits and an inhibitory domain has been proposed with the C-terminal half. Both the  $\gamma$  and  $\beta$  domains are obligatory for full AMPK enzymatic activity (28,35). The  $\beta$  subunit contains a glycogen-binding domain, a C-terminus necessary for complex formation with the  $\alpha$

and  $\gamma$  subunits, and multiple phosphorylation sites although their corresponding upstream kinases and the effects of phosphorylation at these sites remains a point of science (13,35). The  $\gamma$  subunits have variable N-terminal regions followed by four cystathionine- $\beta$ -synthase (CBS) domains. These domains act in pairs, each pair forming a segment referred to as a Bateman domain. The Bateman domain forms a binding site for AMP. Naturally occurring point mutations in the Bateman domains suggest that an AMP molecule has to be bound to each of them in order for AMPK activation to occur (14,32,35,51).

### 1.10.2 Cellular Distribution of AMPK Isoforms

AMPK is expressed throughout most mammalian cells but the expression pattern of the isoform is tissue-specific. The  $\alpha 1$ ,  $\beta 1$ , and  $\gamma 1$  isoform is the predominant form of AMPK expressed in most cell types. The  $\alpha 2$ ,  $\beta 2$ ,  $\gamma 2$ , and  $\gamma 3$  isoforms are also highly expressed, in skeletal and cardiac muscle cell, while  $\alpha 2$  is also significantly expressed by liver cells (34). The differences in isoform combinations are known to vary some of the AMPK function and the type of  $\alpha$  and  $\gamma$  isoforms determine the degree of dependence that the molecule has on AMP for activation. Thus variations in AMPK isoforms allow for some tissue-specific AMPK functions. In addition to tissue-specific expression of the different isoforms, there are also variations in the cellular localization of the isoforms with a large portion of AMPK with the  $\alpha 2$  isoform being associated with the nucleus while those with  $\alpha 1$  are mainly found in the cytoplasm (28,34).



### 1.10.3 Regulation of AMPK

AMPK is a sensor of cellular energy status and is stimulated by rising AMP to ATP ratio due to ATP depletion. Therefore it is activated by metabolic stresses that either inhibit ATP production (heat shock, ischemia, hypoxia, hypoglycemia, metabolic inhibitors such as arsenate or oligomycin, glucose deprivation) or increase ATP consumption (exercise, muscle contraction) (34,37,56). It is now known that AMPK is also stimulated by signals that do not cause changes in the AMP to ATP ratio (e.g. hyperosmotic stress, metformin) (13).

#### 1.10.3.1 AMPK as an intracellular energy sensor and its regulation by 5'-AMP

As its name implicates AMPK is activated by 5'-AMP. This occurs via three different mechanisms: allosteric activation of the phosphorylated molecule, phosphorylation of Thr172 by upstream kinases and inhibition of dephosphorylation of Thr172 by protein phosphatases (35). This multiple mechanism of activation makes AMPK very sensitive to small changes in the AMP to ATP ratio. AMP is produced in several reactions but the majority is produced by the adenylate kinase reaction:  $2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$ . Healthy cells at rest usually have a high ATP to ADP ratio making the direction of the above reaction favour production of ADP and resulting in low AMP levels. Conversely, cellular situations which result in a depletion of ATP, and therefore a low ATP to ADP ratio, will result in an increase in AMP levels. Decreases in ATP levels will drive an increase in AMP (35,37). Allosteric activation by AMP causes an up to 5-fold increase in activity while phosphorylation of the Thr172 residue by upstream kinases results in more than a 100-fold increase in AMPK activity. Although allosteric activation alone renders

AMPK relatively inactive until phosphorylated by upstream kinases, the phosphorylation process appears to be largely influenced by the binding of AMP to AMPK which causes AMPK to be a better substrate for phosphorylation. The effect of AMP on both phosphorylation and dephosphorylation of AMPK appears to be due to binding of the molecule to AMPK rather than the upstream kinase or phosphatase (51,118). As mentioned earlier each of the Bateman domains found on the gamma subunit bind one molecule of AMP or ATP in a mutually exclusive way (35,51). Consistent with this all three mechanisms of AMP on AMPK are antagonized by high concentration of ATP most likely due to competition between the molecules. In addition to interfering with AMP binding, ATP binding to the AMPK inhibits phosphorylation by upstream kinases and stimulates dephosphorylation by phosphatases. This emphasizes the importance of a rising AMP to ATP ratio as the stimulant for AMPK activation rather than simply a rise in AMP or decrease in ATP on their own (34,37,118).

#### **1.10.3.2 Upstream Kinases of AMPK**

##### **Activation by LKB1**

LKB1 exists as a complex with two other subunits, STE20-related adaptor protein (STRAD), and mouse protein 25 (MO25), and is required for activation of AMPK in response to stimuli that increase AMP or compounds that mimic AMP both in cell culture and skeletal muscle *in vivo*. STRAD, which is a pseudokinase, and MO25, which stabilizes the association of LKB1 and STRAD, are both required for catalytic activity. Localization of LKB1 to the cytoplasm also appears to be dependent on the binding by MO25 and STRAD (32,51,118). The LKB1 is

ubiquitously expressed and appears to be constitutively active. LKB1 activity is not stimulated directly by either upstream stimuli of AMPK or by AMP. Instead AMP binding to AMPK causes AMPK to become a better substrate for LKB1 and thus activate the LKB1-AMPK cascade (32,35,51,118). Before the determination that LKB1 phosphorylates AMPK, LKB1 had no identified physiological downstream target. Recently it has been shown that LKB1 activates 11 AMPK-related kinases by phosphorylating a comparable residue to Thr172 in AMPK. This implies that the function of LKB1 may be that of a master kinase which opens the possibility that some of its actions could be mediated by the AMPK-related kinases. However, the physiological role of these kinases as it relates to LKB1 is not known (32,51).

#### **Activation by CaMKKs**

AMPK can also be stimulated by AMP-independent mechanism which is demonstrated by that the phosphorylation of Thr172 by the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinases (CaMKKs) is not stimulated by increasing AMP:ATP ratio but instead by a rise in calcium (35). Although more work needs to be done to determine the tissue distribution of this pathway, CaMKKs appear to be mainly expressed in neural tissue and muscle-specific knockouts of LKB1 suggest that the calcium-mediated pathway cannot have a significant contribution in skeletal muscle. However, contraction-induced glucose transport in skeletal muscle cells is believed to be in part mediated by increased cytosolic calcium levels and activation of the calcium/calmodulin-dependent protein kinase, and some studies in muscle suggest that a role for CaMKK in muscle (35,49,69).

#### 1.10.4 AMPK Function in Metabolism

Since AMPK is instrumental in maintaining energy balance at a cellular and whole body level it may be important in disorders of energy metabolism such as obesity, Type 2 diabetes, and metabolic syndrome. Consistent with this idea, AMPK activation causes many metabolic changes that would be beneficial in subjects with these conditions including increased glucose uptake and metabolism by muscle and other tissue, decreased glucose production by the liver, and decreased synthesis and increased oxidation of fatty acids (28,34,35,51). AMPK plays a role regulating energy intake, utilization, and storage by affecting the intake of food and metabolism. To do this it mediates the action of many molecules and pathways of skeletal muscle, heart, adipose tissue, liver, pancreatic  $\beta$  cells, and the brain which regulate glucose and lipid uptake, storage, and utilization (51). In general AMPK activation inhibits ATP consuming pathways, particularly biosynthetic pathways in order to conserve ATP, and turns on ATP generating pathways. This is achieved by its acute effects as a kinase resulting in phosphorylation of enzymes and by chronic effects on gene and protein expression (51). In transgenic animal models deletion of both  $\alpha 1$  and 2 subunits results in a phenotype incompatible with life, deletion of only  $\alpha 1$  does not present with any observable metabolic phenotype, and deletion of  $\alpha 2$  results in a phenotype with mild insulin-resistance and an insulin-secretory defect. Consistent with a role of AMPK in the hypothalamus, feeding the  $\alpha 2$  knockout mice with a high fat diet leads to weight gain and fat mass compared to controls (51). In view of its regulating role of energy metabolism AMPK has become a potential target for treatment of type 2 diabetes and insulin resistance.

#### **1.10.4.1 The role of AMPK in Skeletal Muscle Glucose Transport**

There are many studies implicating AMPK in glucose uptake in skeletal muscle. The initially studies were done using 5-aminoimidazole-4-carboxamide ribose (AICAR). AICAR is a nucleoside that is taken up into cells and converted to an AMP analogue, AICA riboside monophosphate (ZMP), which mimics the effects of AMP on the AMPK system resulting in AMPK activation (35,84,107). Although AICAR is not specific to AMPK and so results of these studies need careful scrutiny, the studies were able to show that AICAR stimulates glucose uptake in skeletal muscle cells and that AMPK is indispensable for this stimulation (28,29,42,84). Due to the limitation of AICAR studies transgenic and knockout animal models have been important in furthering the understanding of the role of AMPK in skeletal muscle glucose uptake. In both, the whole body AMPK $\alpha$ 1 and whole body AMPK $\alpha$ 2 knockout mice the contraction-stimulated glucose transport was shown to be normal but there was also a noted two to three-fold increase in  $\alpha$ 1 expression and a 2-fold increase in contraction-induced  $\alpha$ 1 activity that might have compensated for some  $\alpha$ 2 functions and explain the observed normal glucose uptake (28,29). In transgenic mice expressing a dominant-negative AMPK $\alpha$ 2 isoform in skeletal muscle AICAR and hypoxia-stimulated AMPK activity and glucose transport were completely abolished, while contraction-stimulated glucose transport was decreased by only 30-40% (29). This further provides evidence that AMPK activity is necessary for AICAR's effect on glucose transport, but shows that it is only partly involved in the contraction stimulated glucose transport. Recently two LKB1 knockout models were generated: a muscle specific LKB1 knockout mouse, and a LKB1 mouse that has a 70-80% loss of

LKB1 in all body tissues and complete ablation of LKB1 in skeletal muscle. These mice showed partial inhibition of contraction-stimulated glucose transport which could not be explained by a decrease in force production during contraction. These results illustrate that LKB1 is necessary to generate a full contraction-stimulated glucose uptake in skeletal muscle cells but that other signals are also required in regulating glucose transport via this stimulus (29).

#### **1.10.4.2 Downstream Targets of AMPK in Glucose Uptake**

The signaling pathway or direct target(s) of AMPK-mediated glucose uptake in skeletal muscle has not been elucidated. It has been shown however that in cell lines that only express GLUT1 transporters (clone 9 cells, 3T3 L1 pre-adipocytes, and C2C12 myoblast) AMPK activation increases glucose transport but does not alter GLUT1 content in the plasma membrane suggesting that AICAR may stimulate the intrinsic activity of existing plasma membrane GLUT1 transporters (28,29,84). In incubated papillary muscle cardiomyocytes and perfused rat hindlimb muscles AICAR-stimulated glucose uptake leads to increased GLUT4 translocation, and H-2Kb cells overexpressing constitutively active AMPK show an increase in GLUT1 and GLUT4 translocation. Based on these studies it is likely that AMPK activity increases glucose uptake in skeletal muscle cells by mediating glucose transporter translocation (84). The downstream molecules involved in glucose transport and glucose transporter translocation by AMPK have not been clearly identified but there has been extensive research and some suggestion have emerged including Akt substrate 160 kDa (AS160), p38, and the sequential activation of the extracellular signal-regulated kinase (ERK), proline-rich tyrosine kinase-2 (PYK-2), phospholipase

D (PLD) and by atypical protein kinase C (aPKC) isoforms (29). The strongest evidence implicates AS160. There are some similarities between the AMPK and Akt substrate motifs and it has been shown that both molecules phosphorylate 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase on Ser466. These facts suggest the possibility that AS160 may also be a shared substrate (29,113). In a study with AMPK $\alpha$ 2-inactive transgenic mice it was shown that AICAR-stimulated phosphorylation of AS160 was completely inhibited (29). Recently Thong *et al* provided evidence that AS160 may be the point of convergence mediating GLUT4 trafficking by various signals including Akt, PKC, and AMPK (113).

AMPK has also been implicated in regulating some effects of insulin and consistent with this, AMPK $\alpha$ 2 null mice have impaired insulin sensitivity (13,35). The mTOR/S6 kinase 1 (S6K1) pathway is implicated in affecting insulin sensitivity. Activation of the mTOR/S6K pathway results in inhibition of insulin signaling by affecting IRS and PI3K, which prevents the normal activation of the pathway by insulin and may lead to development of insulin resistance (51). Specifically, S6K1 phosphorylates the IRS proteins on serine residues, which prevents IRS tyrosine phosphorylation, and S6K1 decreases IRS protein levels by affecting its transcription and protein degradation. Mice with an inactive S6K1 are protected against the development of insulin resistance. The pathway regulating S6K1 activity includes the tumor suppressor products Tuberous Sclerosis Complex (TSC) proteins 1 and 2. TSC proteins regulate the activity of mTOR by increasing Rheb-GDP levels, which reduce both mTOR and S6K1 activation (51). S6K1 is inhibited by being phosphorylated on Thr389 and AMPK has been shown to cause such

phosphorylation. Furthermore the signaling pathways of both AMPK and PI3K/Akt regulate TSC. Whereas the PI3K/Akt pathway functions to prevents TSC activity leading to the activation of the mTOR pathway, AMPK phosphorylates and activates TSC leading to inhibition of mTOR. Another mechanism by which AMPK may blocks mTOR is by directly phosphorylating it (51). There is also evidence suggesting that AMPK may interact with molecules in the insulin signaling pathway. In cell-free assays AMPK phosphorylates IRS-1 on Ser789, an effect also observed in C2C12 myotubes with AICAR-induced AMPK activation, and in myotubes the phosphorylation is also associated with increased binding of PI3K to IRS-1 (37,84,108).

### **1.11 Diabetes Mellitus and Insulin Resistance**

Diabetes mellitus is a chronic metabolic disease and the number of people afflicted by it has been greatly increasing over the past 20 years and continues to increase due largely to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (73,98). The worldwide prevalence of diabetes for all age groups in 2000 was estimated to be 2.8% and is expected to rise to 4.4% by 2030. The estimated number of people  $\geq 20$  years of age with diabetes in 2000 was ~171 million. Assuming that the prevalence of obesity remains the same this number is expected to more than double by 2030 to 366 million (98). In Canada 1.4 million people have been estimated to have diabetes and this number is expected to increase to 2.4 million in 2016. This would indicate a 72% increase in the number of cases compared to an estimated population increase of 12%. This disease has a great economic burden in Canada with 2000 cost estimates being 4.66 billion and



with a projected increased costs of 8.14billion by 2016 (90). In Ontario between 1995 and 2005 the prevalence of diabetes has increased from 4.9% to 8.9%. Notably, although in both, the 1995 and 2005 estimates, the age adjusted prevalence is higher in the older age groups, the prevalence of diabetes among the younger age group has increased by 31% more than in the older age group between the ten years (73).

Diabetes is a condition in which the body is unable to adequately produce and/or use insulin. Insulin is critical in the regulation of glucose and fat metabolism as it has numerous metabolic functions in various tissues including stimulating glucose uptake into skeletal muscle and adipose tissue, stimulating lipid synthesis in adipose cells and liver, inhibition of glucose production and release from the liver, and inhibition of the release of fatty acids from triglycerides in adipose and muscle (5,63,99). As such, proper production and function of insulin plays an important role in controlling the switching between energy fuel utilization in order to meet the body's challenge of continuously supplying adequate energy to all of its cells in the face of irregular supply and ever-changing demand. Under normal conditions insulin promotes glucose utilization for energy by the body while the lack of insulin leads to fat utilization and restricts glucose utilization to those tissues that normally can only utilize glucose, such as the brain. This regulation maintains blood glucose levels in a narrow range in the unfed state. A principle mechanism controlling insulin release into the blood and thus this control is blood glucose concentrations: high blood glucose stimulating insulin secretion and low concentrations inhibiting insulin secretion. In diabetes mellitus blood glucose concentrations are unable to stimulate proper metabolic regulation by insulin and fail to maintain the normal range of blood

glucose levels, resulting in an abnormal metabolism of glucose and fat in various tissues (5,63,99,102,123). The deregulation of metabolism may lead to chronic complications such as retinopathy and blindness, neuropathy, nephropathy, amputations, and accelerated atherosclerosis (82,83,110). Based on Health Canada classification (43) patients are diagnosed with diabetes if they meet one of three criteria:

1. They exhibit symptoms of diabetes such as fatigue, excessive thirst, excessive urination and unexplained weight loss, and have a casual plasma glucose level of  $\geq 11.1$  mmol/L
2. They exhibit a fasting plasma glucose test of  $\geq 7.0$  mmol/L
3. They exhibit a plasma glucose level in the 2 hour sample of the oral glucose tolerance test  $\geq 11.1$  mmol/L.

The underlying pathophysiology of diabetes is diverse and the disease occurs in three forms: type 1, type 2, and gestational diabetes (43). Gestational diabetes occurs in some women during pregnancy and usually is relieved after giving birth, although this condition is associated with an increased risk for developing diabetes later in life (43). Type 1 diabetes, also known as insulin-dependent diabetes mellitus, is characterized by autoimmune destruction of the pancreatic  $\beta$  cells resulting in insufficient or complete lack of insulin secretion (110,123). The prognosis for subjects with this form of diabetes significantly improved after the discovery and purification of insulin between 1921-1922 by the efforts and contributions of Canadian researchers Frederick G. Banting, Charles H. Best, John J.R. Macleod, and James B. Collip at the University of Toronto (100). Daily injections of insulin at

appropriate times in these subjects helps maintain proper blood glucose levels and energy metabolism and delay the development of diabetes-related complications. Type 2 diabetes is the most common form of diabetes accounting for ~90% of cases (110). In type 2 diabetes the abnormal fat and glucose metabolism is usually due in part to resistance of skeletal muscle, liver, and fat to insulin action, and is highly correlated with obesity. There is an increase in glucose production and output by the liver and impaired disposal of glucose via insulin-stimulated glucose transport. At first the  $\beta$  cells compensate for insulin resistance and maintain relatively normal blood glucose levels by secreting more insulin. However, in the natural history of the disease there is a gradual progressive  $\beta$  cell failure to secrete enough insulin to compensate for peripheral insulin resistance in the later stages of the disease (77,83)

Both environmental and genetic factors are attributed to the development of type 2 diabetes and although the primary defect is unknown, insulin resistance in skeletal muscle has been established as an early defect in the pathogenesis of type 2 diabetes. Other factors such as hyperglycaemia, hyperinsulinemia, and other metabolic irregularities may further diminish insulin action and contribute to the development of insulin resistance (63,123). Despite it being suggested that type 2 diabetes begins with skeletal muscle insulin resistance studies with transgenic tissue-specific knockout mice show that skeletal muscle insulin resistance may not on its own lead to the development of the disease (5,123). Saltiel *et al* (102) suggests a unifying hypothesis for the development of type 2 diabetes in which “insulin resistance in classic target tissues (liver, muscle, fat) coupled with insulin resistance in  $\beta$ -cells, brain and other tissues, combine to produce the pathophysiology of type 2

diabetes". In recent years it has become recognized that in the pathophysiology of type 2 diabetes  $\beta$ -cells failure occurs earlier than previously suspected, and to involve problems with skeletal muscle, adipose, liver, gastrointestinal track,  $\alpha$ -cells of the pancreas, kidney, and brain (19.1).

Insulin resistance refers to a reduced responsiveness of tissues to circulating concentrations of insulin and is a consistent feature of type 2 diabetes. This however underscores the heterogeneity of the insulin resistance phenotype, which may vary depending on which tissues are affected and which insulin pathways within those tissues are defective and to what degree (5). At a molecular level insulin resistance has been associated with several defects in the insulin signalling pathway including decreases in receptor concentration and kinase activity, the concentration and phosphorylation of IRS-1 and -2 proteins, PI3K activity, and glucose transporter translocation (102). Particularly insulin resistance in the skeletal muscle of obese and type 2 diabetic patients is associated with decreases in insulin receptor tyrosine kinase activity, decreased IRS-1-associated tyrosine phosphorylation and insulin-stimulated PI3K activation. Adipose tissue of obese individuals show a decrease in basal and insulin-stimulated 2-deoxyglucose uptake, a decrease in the number of insulin receptors, and a decrease in insulin receptor tyrosine kinase activity and IRS-1 associated PI3K activity. In the liver of obese individuals there is no significant change in the number of insulin receptors, insulin receptor kinase activity, or in insulin's ability to inhibit glycogenolysis (5).

Presently there is no cure for diabetes mellitus as is true for many of the major chronic diseases inflicting large segments of the world population. There is a

multifactorial theory of current major diseases but we have not yet a clear paradigm shift in our understanding of chronic diseases such as diabetes mellitus although major advancements have been made. This means that the focus for the diabetic patient is life-long treatment regiments aimed at mimicking normal metabolic control and delaying associated complications, and prevention strategies for the general population to reduce the risk for development of the disease. For the type 2 diabetic this means adapting strategies for managing the condition and reducing the risk of complications such as healthy diets, weight management, regular physical activity, and/or using oral medications. Currently there are a variety of different classes of oral medications available for treatment of type 2 diabetes that differ in their targets and mechanism of action. These include drugs that stimulate insulin release from the  $\beta$  cells (sulfonylureas, meglitinides); inhibit glucose absorption by the gut ( $\alpha$ -glucosidase inhibitors); reduce hepatic glucose output through inhibition of gluconeogenesis and increase peripheral glucose transport (metformin); reduce insulin resistance at target tissues (thiazolidinediones or glitazones) (77,82). Given that the current strategy for therapy is management, the complexity and variety of the possible defects, and that as a result the current drugs have limited efficacy in certain patients due to their specific mechanisms, it is important to study novel compounds that may bypass insulin resistance by having an insulin-like effect by stimulating specific insulin signalling pathways or other pathways with similar effects, or by increasing insulin sensitivity, thus possibly leading to improved treatment options for type 2 diabetes. The study of such novel compounds and their mechanism(s) of action

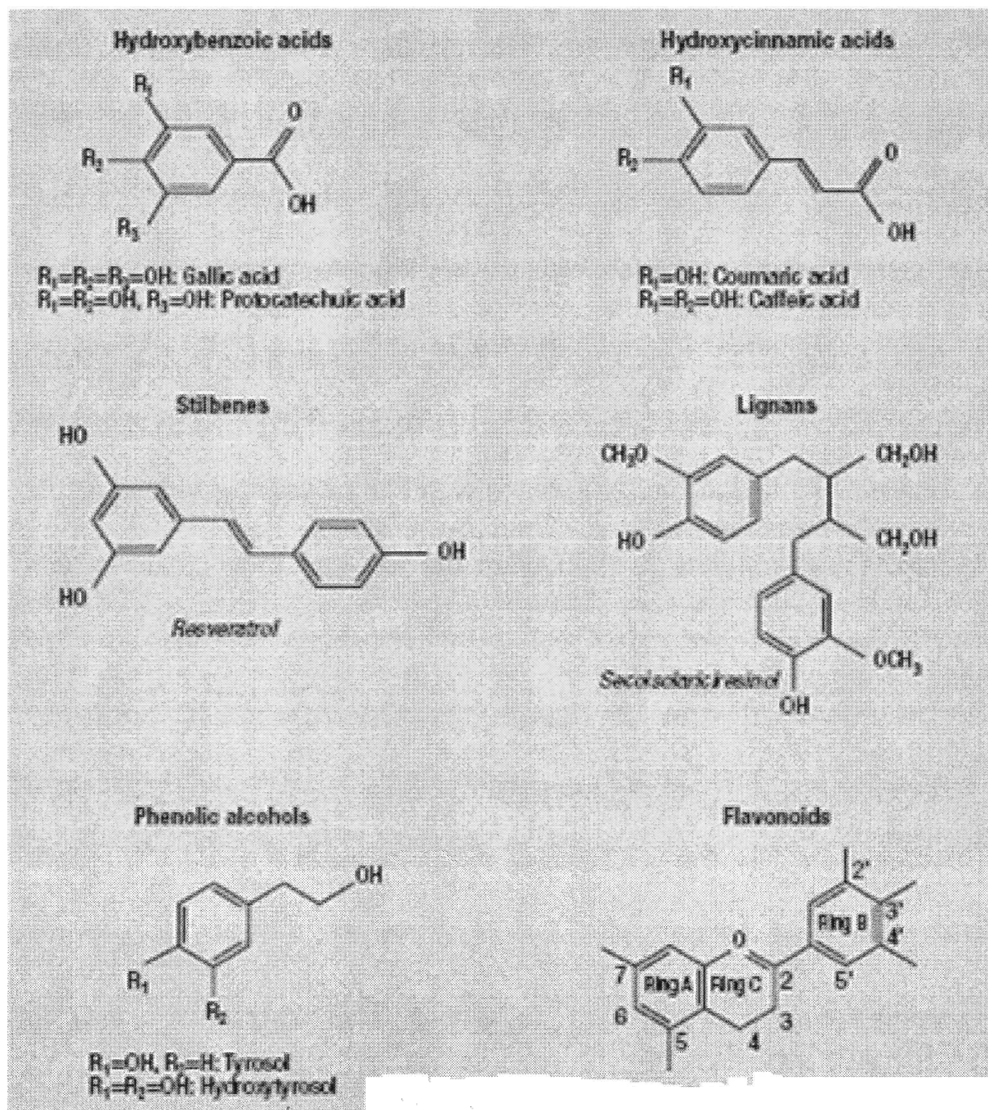
also have indirect benefits in that they may lead to further elucidation of signalling pathways involved in metabolism, their cross talk, and unifying factors.

### **1.12 Polyphenols**

Epidemiological studies suggest that a diet high in fruits and vegetables is protective against chronic diseases such as cardiovascular disease and cancer but the role played by individual components of these foods in this protection and to what extent they account for the decreased risk of disease is difficult to determine (24). Polyphenols have increasingly gained attention from the scientific community for their potential health benefits and as therapeutics against several chronic diseases. The main reason for the interest in polyphenols is due to recognition of them as the most abundant antioxidants of our diet and their abundant distribution in food and beverages of plant origin such as fruits, vegetables, cereals, legumes, chocolate, tea, and wine. The distribution of specific polyphenols in food varies with some being highly ubiquitous (e.g. quercetin) while others being specific to certain foods (e.g. naringenin in citrus fruit) (19,75). Apart from being constituents of a regular diet polyphenols are emerging as potential active components of certain traditional folk remedies (72,75). Polyphenols have been reported to have antioxidant properties and may protect against diseases including cancer, cardiovascular disease, neurodegenerative diseases, and diabetes. Whether the beneficial health effects are due to antioxidant activity is not clear as some studies have shown pro-oxidant effects as well (19,31.1). Increasingly it is becoming known that in addition to their antioxidant properties, polyphenols possess a variety of other specific biological effects, such as regulation of enzymes, and may prevent diseases by mechanisms that

are independent of their antioxidant effects. The most extensively studied polyphenol is the stilbene resveratrol (Figure 4) which has been reported to have anticancer, anti-cardiovascular disease, and anti-diabetic properties. Resveratrol is an activator of the protein deacetylases called sirtuins. Mainly studies indicate that in mammalian tissue SIRT1, one of the members of sirtuins, may be involved in the biological effects of resveratrol (19,46, 47, 65, 106).

With over 8000 polyphenolic structures having been identified polyphenols are one of the most widely distributed groups of compounds in plants (101). They are produced as secondary metabolites in plants and are involved in plant growth and reproduction, resistance to pathogens, protection from ultraviolet light, pigmentation, and flavor (75,101). Despite the name, polyphenols have the common feature of an aromatic ring bearing *at least one* hydroxyl group although commonly they have several hydroxyl groups on aromatic rings. They can be divided into several different classes according to the number of phenol rings they contain and the structural elements binding the rings together. The main classes of polyphenols are phenolic acids, stilbenes, flavonoids, lignans, and phenolic alcohols (Figure 4) (19,75). In addition to their diversity in chemical structure they can also exist as either bound to various sugars (glycosilated) or unbound (aglycons) (75).



**Figure 4:** The different classes of polyphenols. Taken from ref 19.

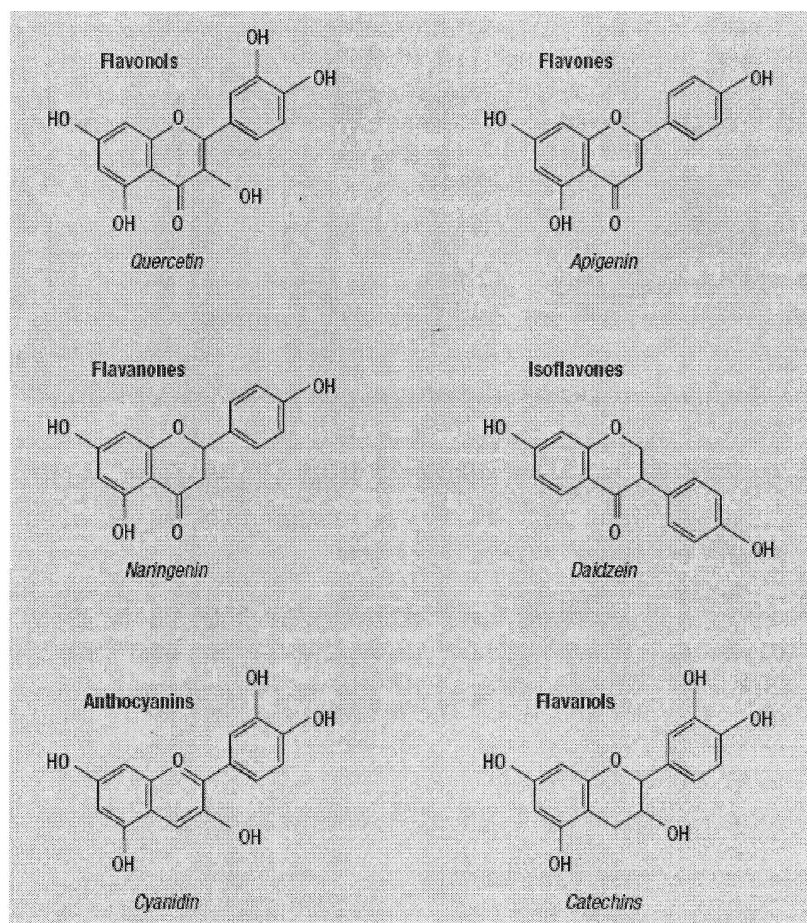
### 1.12.1 Flavonoids

Flavonoids are the largest class of polyphenols, with over 5000 flavonoids being identified, and are potent antioxidants *in vitro* (101). They share the common structure of 2 aromatic rings that are joined by a linear 3 carbon chain (C6-C3-C6) that forms an oxygenated heterocycle, and can be divided into 6 subclasses depending



on the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (Figure 5) (75). Naringenin, the focus of the present work, belongs to the flavanone subclass of flavonoids, which are characterized by a saturated 3-carbon chain of the heterocycle and an oxygen atom at carbon 4 (Figure 5) (19). In plants flavonoids are usually present as glycosides and the flavanone subclass usually exists in foods bound to either a neohesperidose or a rutinose (75). High concentrations of flavanones in the human diet are only present in citrus fruit although they are also found in tomatoes and some aromatic plants such as mint. The dietary intake of flavanones are quite high in individuals consuming citrus fruit regularly and the glycoside forms of these compounds are present in high concentrations in many commonly ingested citrus fruits and juices. The main representatives of flavanones are naringenin, hesperetin, and eriodictyol, which are found in highest concentrations in grapefruits, oranges, and lemons, respectively

(19,75,101).



**Figure 5:**  
The different  
subclasses of  
flavonoids. Taken  
from ref 19.

## 1.12.2 Naringenin

### 1.12.2.1 Naringenin Food Sources and Bioavailability

It should be noted that several factors might influence dietary intake and bioavailability of flavonoids, which may also be true for naringenin. For one plant production of flavonoids is influenced by many factors including light, plant genetics, environmental conditions, germination, degree of ripeness, processing and storage, and species variety (101). Also whether the flavonoid is formed glycosylated or not, and its dietary source may affect bioavailability (101). The main food sources of naringenin are grapefruits, oranges, and tomatoes, with grapefruits by far containing the highest concentrations. Naringenin from citrus fruit is principally present in glycosylated forms: in oranges the main naringenin glycoside is narirutin (naringenin-7-rutinoside) while in grapefruit narirutin is present to a lesser extent (20%) and the main form is naringin (naringenin-7-neohesperidoside) (70%). In tomatoes (*Lycopersicum esculentum*) naringenin is one of the most abundant polyphenols, and is present in the skin as aglycon (12,22,24).

There have been limited studies ascertaining the dietary intake of naringenin and given the before mentioned factors that influence food content of polyphenols and bioavailability it is difficult to extrapolate results to other populations. Furthermore it should be kept in mind that a considerable inter-individual variation in naringenin uptake and metabolism has been reported in the results. In Finland the average intake of naringenin is estimated to be 8.3 mg/day while in another study the daily intake in a high-vegetable/fruit diet was 29 mg with a mean plasma naringenin concentration of 112.9 nmol/L (a range of 40-2120 nmol/L in 22% of the individuals

taking naringenin) (23,24). Naringenin content of grapefruit juice has been reported to be 1283  $\mu\text{mol/L}$  with a resulting plasma concentration range of 0.7-14.8  $\mu\text{mol/L}$ ; in orange juice content of naringenin has been reported to be 141  $\mu\text{mol/L}$  with plasma concentrations of 0.1-1.2  $\mu\text{mol/L}$ ; and naringenin concentration of a tomato has been reported to range from 0.8-4.2 mg/100g in whole red tomato, and after ingestion of a tomato paste test meal containing 3.81 mg of naringenin the peak plasma concentration resulted in 0.12  $\mu\text{mol/L}$  (12,22,23) Taken together in all the above mentioned studies the plasma concentration of naringenin in individuals consuming naringenin-rich foods is in the  $\mu\text{M}$  range (Table 1).

**Table 1:** Plasma Concentration of Naringenin in Humans after ingestion from food sources.

Food Source	Amount of Naringenin ingested	Plasma Concentration	Reference
Orange Juice, half orange, half mandarin	29 mg	112.9 nmol/L <sup>a</sup>	23
Orange Juice	85 $\mu\text{mol}$ 23 mg	0.1-1.2 $\mu\text{mol/L}$ <sup>b</sup> 175 $\mu\text{g/L}$	22
Grapefruit Juice	731 $\mu\text{mol}$ 199 mg	0.7-14.8 $\mu\text{mol/L}$ <sup>c</sup> 1628 $\mu\text{g/L}$	22
Tomato Paste	3.81 mg in tomato paste	0.12 $\mu\text{mol/L}$ <sup>d</sup>	12

a - mean. Large number of subjects had values below detectable via method.

b,c - values are range and mean peak plasma concentrations, respectively.

d - mean peak plasma concentration.

In general polyphenols follow a common pathway of metabolism. Both the aglycones and glycosides can be absorbed in the body. The aglycones can be absorbed from the small intestine and pass passively into the blood stream from the

gut but the glycosides are more hydrophilic and may require transporter proteins and/or to be hydrolyzed by intestinal enzymes or colonic microflora in order to be absorbed (75,94). Peak naringenin concentration are reported to be reached at 5.5h and 4.8h for grapefruit juice and orange juice, respectively, which according to the researcher suggests that naringenin from these juices is absorbed from the distal parts of the small intestine or the colon (22). During absorption polyphenols are conjugated in the small intestine and the liver, which includes methylation, sulfation, and glucuronidation, and the resulting conjugates are largely bound to albumin in the circulation. The effect of albumin binding on the biological activity of polyphenols is however unclear (75). In the study examining the bioavailability of naringenin from tomato paste only conjugated naringenin was detectable in plasma samples suggesting that naringenin is extensively metabolized in the liver from where it leaves to the general circulation in the conjugated form (12).

The elimination of polyphenols mainly occurs in urine and bile, and via the biliary route they may be again reabsorbed (75). Compared to other flavonoids, flavanones have short half-lives although the results of urinary excretion have varied in different studies. The urinary recovery of naringenin has been reported as 5-59%, 5%, 14-15%, and 1-6% in different studies (22). Erlund *et al* (23) found that 63% of total urinary excretion of naringenin was excreted in a 4-8h fraction from grapefruit juice and 83% from orange juice (23). Fuhr *et al* (reviewed in 22) found that the half life for naringenin and naringenin glucosides after ingestion of grapefruit juice was 2.9 and 2.6 h, respectively, while in a study by Erlund *et al* (23) the half life was 1.3-

2.2 h. The amount of excreted of naringenin appears to be dependent on the serving sizes ingested (22,23).

#### **1.12.2.2 Biological Effects**

Intake of citrus has been proposed to have several beneficial properties including anticarcinogenic, antiviral, and anti-inflammatory and epidemiological studies have shown that the consumption of citrus fruits or juices, which contain high amounts of naringenin, have a protective effect on the risk of ischemic stroke and different cancers (23,24). Furthermore epidemiological studies are reported to show that intake of tomatoes and tomato-based products have an inverse correlation with risk of certain cancers (12). There are however limited studies on the impact of specific flavonoid intake on disease incidence partly due to the large variety of the compounds in foods and the complexity of collecting accurate data on food intake and on intake of specific flavonoids. This helps to explain the inconclusiveness of epidemiological studies on the effects of specific flavonoids on health with some studies supporting reduced risks of chronic diseases such as cancer and cardiovascular disease with higher intake, some reporting no effect, and others still a potentially negative effect. Most epidemiological studies have focused on flavones and flavonols intake but not many have examined the impact of flavonones (59). In one study which measured the intake of a variety of flavonoids including naringenin it was observed that a high intake of certain flavonoids was associated with reduced risk of mortality from ischemic heart disease, incidence of cerebrovascular disease, incidence of specific cancers and asthma, and a trend towards a decreased risk for type 2 diabetes. Naringenin intake was associated with a reduced risk of

cerebrovascular disease (59). The study was limited by small number of cases of certain diseases and in that intake was measured by questionnaires and no biomarkers of the different flavonoids were measured making the data subject to recall bias. However because naringenin is found in foods that are a main ingredient (i.e. grapefruit vs. onions which would be considered a hidden ingredient as it is often added to dishes and eaten as part of a mixture) the food intake is expected to have been fairly accurately measured (59). Although the amount of flavonoids in sample foods was measured in the study the absorption and bioavailability of the compounds was not (59).

Studies specifically looking at the effects of naringenin have reported antioxidant, anti-proliferative, and anticancer properties. *In vitro* studies using different cell lines have identified naringenin as an effective antioxidant and *in vivo* naringenin partially protects against oxytetracycline-induced oxidative stress in rat liver (1,22,92). However, in the weanling rat strain exposed to oxidative stress diets naringenin did not exhibit antioxidant effects (1). Other biological activities attributable to naringenin include anti-inflammatory actions and effects on sex hormone metabolism (22). Also naringenin inhibits the cytochrome P450 enzyme and therefore may increase the concentration of certain drugs that are metabolized by this enzyme (22,24). Other effects of naringenin may be on lipid and glucose metabolism (discussed below).

#### **1.12.2.3 Effects of Naringenin on Metabolism: Animal Studies**

A variety of herbal medical preparations traditionally used for the treatment of diabetes exist and their extracts have been tested and shown to possess antidiabetic

properties. Of these extracts several have been identified to contain flavonoids but only in some cases have the specific flavonoid composition been elucidated. *Cochlospermum vitifolium* (Willd.) Sprengel, also known “panicua”, is a tree found in Mexican which bark is used in traditional medicine for the treatment of hypertension, type II diabetes, and hepatitis. Not until recently have there been reports of isolated active compounds from this preparation with vasorelaxant, hypoglycaemic and hepatoprotective activities (103). A study by Sanchez-Salgado *et al* (103) aimed to confirm the antidiabetic and antihypertensive properties of this preparation. They tested hexane, dichloromethane, and methanol extracts from the preparation for their vasorelaxant, hypoglycaemic, and hepatoprotective activities in male Wistar rats. Chromatography fractionation of the methanol extract isolated naringenin. The study found that the methanol extract and naringenin exerted vasorelaxant activity and the methanol extract showed a hypoglycaemic effect but which did not reach statistical significance. Unfortunately the researchers did not specifically test the hypoglycaemic potential of naringenin in this study (103).

Studies in rodents have shown that naringenin possesses hypocholesterolemic and hypoglycaemic effects, and improves hyperlipidemia and atherosclerosis. In a study examining the hypoglycaemic and hypolipemic properties of naringenin isolated from stems of *Prunus davidiana*, a Korean folk medicine for neuritis and rheumatism, in streptozotocin-induced diabetic rats the researchers found that intraperitoneal administration of naringenin (10 mg/kg body weight) significantly decreased plasma glucose concentrations by 10%, decreased triglycerides levels by 61%, and decreased total cholesterol levels by 25% (17). In another study the same

researchers examined the effects of naringenin on serum parameters in high-fat fed rats (18). In the study naringenin was shown to exhibit a significant hypocholesterolemic effect with the naringenin treated group (20 mg/kg B.W.) resulting in a significant 9% decrease in total cholesterol. A study by Santos *et al* (104) examined the effect of naringenin on lipid metabolism in hyperlipidemic rats and found that naringenin significantly reduced cholesterol levels and triacylglycerol levels.

A study by Lee *et al* (70) provides some elucidation into the mechanism of the cholesterol lowering effect of naringenin in which high-cholesterol fed rats were supplemented with or without naringenin. The study found that naringenin did not alter plasma triglyceride levels but significantly reduced plasma cholesterol levels and hepatic cholesterol content. The effects on two key enzymes, acyl-CoA:cholesterol acyltransferase (ACAT) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase), involved in cholesterol metabolism were also examined. ACAT is involved in the formation of cholesteryl esters, cholesterol absorption from the diet, hepatic secretion of very low-density lipoprotein (VLDL)-cholesterol, and in the development of foam cells in atherosclerosis (70). HMG-CoA reductase is a rate-limiting enzyme of cholesterol formation (68). This study found that naringenin supplementation reduced the activity of both of these enzymes (70). In another study the anti-atherogenic effects of naringin and naringenin in high cholesterol-fed rabbits was examined (68). The animals that were fed a 1% cholesterol diet supplemented with naringin or naringenin showed reduced activity of hepatic ACAT by 5% and 15% respectively, a reduction of the aortic fatty streak areas by 19.2% and 18.1%



respectively, and reduced expression levels of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1). Adhesion of monocytes to the endothelium is an early step in the development of atherosclerosis and VCAM-1 is suggested to be involved in the recruitment of monocytes in this condition. MCP-1, in addition to recruiting monocyte, also promotes the release of lipids and lysosomal enzymes into the extracellular space which leads to the progression of atherosclerosis (68).

The hypoglycemic effects of naringenin *in vivo* have been less studied. A study by Li *et al* (71) examined the effects of naringenin on intestine and renal brush border membrane vesicles (BBMV) of normal rabbits (intestine BBMV only) and normal and STZ diabetic rats. The study found that glucose uptake activity in the intestine BBMV of rabbits was moderately inhibited by naringenin. However glucose uptake in intestine and renal BBMV of STZ diabetic rats was significantly higher than in normal rats and naringenin (500  $\mu$ M) normalized this uptake to levels similar to control rats. Naringenin decreased glucose uptake in both diabetic and normal rats by ~80% in intestinal BBMV and by ~60 in the renal BBMV (71).

#### **1.12.2.4 Effects of Naringenin on Metabolism: Cell Culture Studies**

There are limited studies examining the metabolic effects of naringenin in cell culture. There have been a few studies examining the mechanism of the hypolipidemic effects of naringenin in hepatocytes HepG2, and naringenin has been shown to inhibit apolipoprotein B (apoB) secretion in these cells, an effect also observed by insulin (7,8,9). Hepatic overproduction of apoB-containing lipoproteins (apo-Lp) is associated with a number of hyperlipoproteinemias. ApoB provides the

structural framework for the formation of very low density lipoproteins (VLDL) (7). Prior to their secretion, apoB associates with lipids in the endoplasmic reticulum (ER), initially acquiring small amounts of phospholipids, triglycerides (TG), and cholesteryl ester (CE), and as the protein is further translated and translocated into the rough ER more lipids are acquired (7). The synthesis of CE and TG is performed by ACAT and diacylglycerol acyltransferase (DGAT), respectively, and the microsomal triglyceride transfer protein (MTP) transfers the synthesized lipids to apoB. MTP is necessary for lipoprotein formation and secretion (7). Research by Borradaile *et al* has shown that the naringenin-stimulated reduction in apoB secretion is associated with reduced ACAT and MTP activities and reduced expression of MTP and ACAT2 (the form of ACAT expressed exclusively in liver and intestine). It has also been shown that naringenin inhibited accumulation of both TG and CE in the microsomal lumen suggesting that as a consequence the availability of lipids for the assembly of hepatic apoB-Lp is inhibited. Notably newly synthesized CE within microsomal lumen, secondary to ACAT activity, does not seem to be the means of inhibiting apoB secretion. Naringenin was also shown to increase intracellular degradation of apoB. Ultimately these studies lead to the determination that naringenin inhibits the apoB-Lp assembly and subsequent secretion of apoB-containing lipoproteins by limiting the accumulation of TG in the ER via MTP inhibition (7,8).

Insulin is known to inhibit ApoB secretion in hepatocytes by activating PI3K which leads to increased sterol regulatory element-binding protein (SREBP)-1 and LDL receptor expression. LDLr activity can regulate net apoB secretion by directing uptake of the newly secreted apoB-Lp and leading nascent apoB-Lp to degradation

(9). In further elucidating the inhibitory effect of naringenin on apoB secretion Borrandale *et al* (9) examined the effect of naringenin on expression and activity of SREBP-1 and LDLr. The study found that naringenin has an insulin-like effect to stimulate PI3K and causes a PI3K-dependent increase in cytosolic and nuclear SREBP-1 and LDLr expression. Both naringenin and insulin were also shown to decrease insulin receptor substrate IRS-2 levels although, unlike insulin, naringenin did not stimulate tyrosine phosphorylation of IRS-1 (9).

There are no reported studies of the effects of naringenin on glucose uptake in skeletal muscle cells *in vitro*, but there are a few in other cell types including adipocytes, monocytes, and breast cancer cells. Based on a previous report that the isoflavone genistein worked as an inhibitor of glucose transport but that there remained conflicting reports as to the inhibitor effects of flavonoids on glucose transport, Park (94) examined a variety of flavonoids with respect to their glucose transport blocking potential in U937 monocytes. The study found that several of the flavonoids blocked glucose uptake with various efficacy the most potent of which, fisetin, blocking ~half of the glucose uptake with 8uM. Naringenin exhibited a relatively weak inhibitory effect on glucose uptake with a 50% reduction in basal glucose uptake requiring approximately 50  $\mu$ M naringenin. Harmon *et al* (40) examined the effect of naringenin on glucose uptake in 3T3-L1 adipocytes and the study found that naringenin inhibited insulin-mediated glucose transport is a dose-dependent manner with 100  $\mu$ M naringenin inhibiting glucose uptake by ~85%. In the study naringenin did not affect the phosphorylation of the insulin receptor, IRS-proteins, or PI3K, but inhibited the phosphorylation of Akt (40). In an *in vitro* PI3K

activity assay, naringenin blocked production of  $\text{PIP}_3$  by PI3K, an effect similar to that of wortmannin. In the same study 6 $\mu\text{M}$  naringenin, a physiologically obtainable concentration, was shown to inhibit insulin-stimulated glucose uptake by 20%. In study examining the effects of naringenin on glucose uptake in MCF-7 breast cancer cells naringenin treatment was found to inhibit insulin-mediated glucose uptake in proliferating and growth arrested cells (39). Breast cancer cells overexpress GLUT1 and often also express the GLUT4 transporters and experienced increased glucose uptake in response to insulin (39). In this study naringenin inhibited the phosphorylation of Akt of MCF-7 cells and phosphorylation of MAPK. The authors indicate that in this cell line the MAPK pathway seems to play an important role in insulin-stimulated glucose uptake by showing that inhibition of the pathway with a MAPK kinase inhibitor, PD98059, decreased the insulin-stimulated glucose uptake by ~60%. In the MCF-7 cells naringenin was shown to inhibit cell proliferation, a result which was also observed when glucose concentration was decreased in the growth media. Lastly the study showed that a physiologically attainable dose of 10  $\mu\text{M}$  naringenin decreased insulin-mediated glucose uptake by 25% and cell proliferation (39).

## CHAPTER 2: Rationale and Hypothesis

The role of insulin in regulating metabolism is complex as it has diverse actions and affects different types of tissues. The underlying cause of dysfunction that occurs in some people which leads them to develop insulin resistance, and why insulin resistance manifests itself in different ways is not fully understood. Therefore treatment for insulin resistance at present is focused on management rather than cure. Management is difficult given the diverse and coordinated actions of insulin, and present oral treatments are limited in their efficacy due to their specific targets and mechanism(s). The study of the effects of novel compounds on metabolism remains an important area of research both for potentially developing new treatment approaches and options, and in furthering knowledge of metabolic regulation.

A major consequence of insulin resistance is inadequate peripheral glucose disposal. Skeletal muscle tissue is one of the targets of insulin and it is responsible for ~80% of glucose uptake in the post-prandial state making this tissue an important treatment target (53). The flavonoid naringenin has been reported to have antioxidant, antiproliferative, anticancer, and anti-inflammatory properties (1,22,92) and exhibits effects on glucose and lipid metabolism. *In vitro* naringenin has been shown to decrease basal oleate-stimulated apolipoprotein B secretion in hepatocytes (7,8,9). In *in vivo* naringenin exhibited a hypoglycaemic effect in male wistar rats, and in streptozotocin-induced diabetic rats naringenin treatment significantly decreased plasma glucose, triglycerides and total cholesterol levels (18,17). Furthermore naringenin has structural similarities with the extensively researched resveratrol and the many benefits of red wine, a particularly rich source of the compound, may be

attributable to it. Resveratrol has been shown in recent years to have antidiabetic effects. Studies by our group (11) and others (3,1,4,65,93,112.1) have shown that resveratrol increases glucose uptake by skeletal muscle cells and also prevents fatty acid-induced insulin resistance *in vitro* and *in vivo*. There are no studies examining the effects of naringenin on glucose uptake in skeletal muscle cells which is the focus of the present work.

### **Hypothesis**

The hypothesis of the present work is that naringenin has an insulin-like effect of increasing glucose uptake in skeletal muscle cells and/or potentiates the effect of insulin.

### **Specific Aims:**

- 1) Determine the effects of naringenin on basal and insulin-stimulated glucose uptake in skeletal muscle cells.
- 2) Elucidate the mechanism of naringenin-stimulated glucose transport in skeletal muscle cells by examining effects on signalling molecules involved or suspected of being involved in glucose uptake regulation in skeletal muscle.

These aims are addressed by examining the effects of naringenin on glucose uptake, and on signalling molecules including Akt, PI3K, AMPK, SIRT1, and CaMKK.

## CHAPTER 3: RESEARCH DESIGN AND METHODS

### 3.1 Materials

Minimum essential medium ( $\alpha$ -MEM), fetal bovine serum (FBS), trypsin, and antibiotic were purchased from GIBCO Life Technologies (Burlington, ON, Canada). FBS was later purchased from HyClone, ThermoFisher Scientific (Ottawa, ON, Canada). Specific antibodies against Akt, phospho-Akt ser473, phospho-Akt thr308, AMPK $\alpha$ , phospho-AMPK $\alpha$  thr172, HRP-conjugated anti-rabbit secondary antibody, and HRP-conjugated anti-mouse were purchased from New England Biolabs (Mississauga, ON, Canada). ChemiGLO reagents were purchased from VWR (Mississauga, ON, Canada). Polyvinylidene difluoride (PVDF) membranes, molecular weight protein standards, and electrophoresis reagents were purchased from BioRad (Mississauga, ON, Canada). [ $^3$ H]2- deoxy-D-glucose, was purchased from PerkinElmer (Boston, MA). Cytochalasin B (CB), wortmannin, and Compound C were purchased from Calbiochem (Gibbstown, NJ, US). The sirtuin inhibitor Ex527 was purchased from Tocris Bioscience (Ellisville, MO, US). The inhibitor of calcium/calmodulin-dependent protein kinase kinase (CaM-KK). STO-609, was a kind gift from Dr. Bilan (Hospital for Sick Children, Toronto, ON, Canada). The SIRT1 activity assay kit was purchased from BIOMOL International (Plymouth Meeting, PA, US). All other chemicals, including naringenin, nicotinamide, bovine serum albumin, and cold 2-deoxy-D-glucose, were purchased from Sigma Chemicals (St. Louis, MO, US). The parental L6 cells were a kind gift from Dr. A. Klip (Hospital for Sick Children, Toronto, ON, Canada).

### 3.2 Preparation of Buffers and Solutions

#### Glucose Uptake Assay:

HEPES Buffer Saline (HBS) Washing Buffer: 140 mM NaCl, 5mM KCl, 20mM HEPES, 2.5mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>. Dilute with distilled (DD) water and fill to 800 ml, adjust pH to 7.4 and then fill to 1 L with DD water.

0.9% NaCl: Add 0.9g NaCl per 100 ml DD water.

0.05N NaOH: Add 1 ml 5N NaOH to 99 ml DD water.

Total Radioactive Buffer: In HBS buffer dilute 1:1000 hot [<sup>3</sup>H]2-deoxy-D-glucose, and 1-1000 cold 2-deoxy-D-glucose. E.g. for 10 ml of HBS, add 10 µl of hot [<sup>3</sup>H]2-deoxy-D-glucose and 10 µl of cold 2-deoxy-D-glucose.

Non-specific Radioactive Buffer: Use total radioactive buffer and add 1:1000 cytochalasin B.

#### Cell Lysis:

Phosphate buffer saline (PBS) Washing Buffer: 137 mM NaCl , 2.7mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.68mM CaCl<sub>2</sub>, 0.49mM MgCl<sub>2</sub>, add water to 1 L and adjust pH to 7.4.

Sodium dodecyl sulphate (SDS) Sample Buffer: 62.5mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 0.01% bromophenol blue, add 0.05% β-mercaptoethanol before use.

E.g. for a 200 ml 3X SDS sample buffer: 60 ml glycerol, 37.5 ml 1.0 M Tris (pH 6.8), 12 g SDS, pure water to 200 ml, 60mg bromphenol blue.



Cell Lysis Buffer: 20mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1 mM  $\beta$ -glycerolphosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{g/ml}$  leupeptin, add 1 mM PMSF before use and chill on ice.

### **Western Blot Analysis:**

10x Tris-buffered saline (TBS): 24.2 g Tris base, 80 g NaCl, add water to 1 L and adjust pH to 7.6.

Blocking Buffer (for 2 membranes): 7.5 ml 10x TBS, 67.5 ml water, 3.75 g nonfat dry milk, and then add 75  $\mu\text{l}$  Tween-20. Prepare fresh daily.

Primary Antibody Dilution Buffer: 2 ml 10x TBS, 18 ml water, 1.0 g BSA, and then add 20  $\mu\text{l}$  Tween-20. Store at 4°C.

Wash Buffer TBS/T: 1x TBS, 0.1% Tween-20. E.g. 450 ml water, 50 ml 10x TBS, and 500  $\mu\text{l}$  Tween-20.

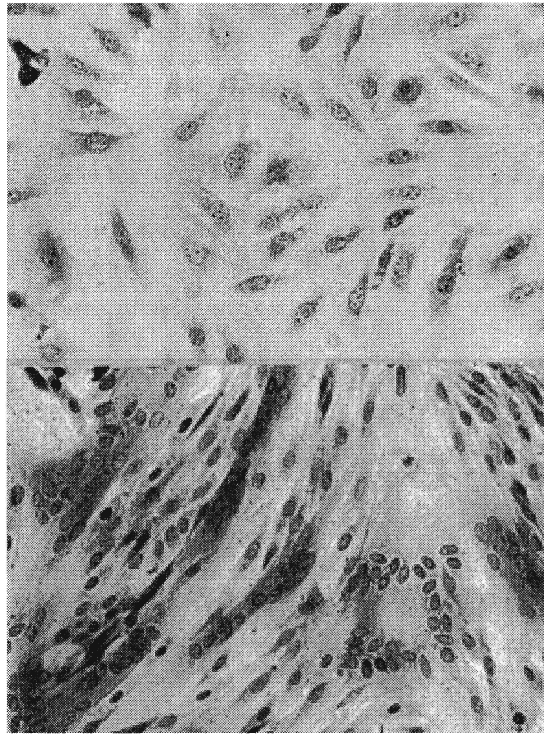
10x Electrode Running Buffer: 15.15 g Tris base, 72 g Glycine, 5.0 g SDS. Dissolve and bring the volume to 500 ml with water. Before use dilute 50 ml of 10x stock with 450 ml water. Store at 4°C.

Transfer Buffer: To make 1 L use 3.03g of 25 mM Tris base, 15.01 g of 0.2 M Glycine, 200 ml of 100% methanol and 800 ml of water. Make fresh daily and chill at 4°C before use.

### **3.3 The L6 Skeletal Muscle Cell Line**

The L6 cell line was prepared from the thigh muscle of newborn rats and consists of mononucleated myoblasts that retain myogenic potential; a characteristic that is inherited by their progeny (125). These myoblasts proliferate at high (10%) serum and can be induced to spontaneously fuse and differentiate into elongated,

multinucleated, postmitotic, contracting myotubes (125) when grown to confluence and in low (2%) serum concentration (6). At the differentiated stage L6 share some of the biochemical, morphological, electrical, contractile, and metabolic characteristics of *in vivo* skeletal muscle (6,58,78). L6 myotubes express the insulin and IGF-1 receptors which exhibit selectivity for their ligands, and other proteins known to be involved in insulin signalling in skeletal muscle including GLUT4 glucose



**Figure 6:** The L6 skeletal muscle cell line. Top: myoblasts. Bottom: Myotubes. Courtesy of Dr Evangelia Tsiani

transporters, IRS-1, PI3K, and Akt (48). The differentiated cells respond to insulin and IGF-1 by an increase in glucose uptake. Acute insulin treatment stimulates glucose uptake with 100 nM insulin treatment reaching a maximal effect of 1.6- to 2-fold above basal within 15 minutes. After the initial response to insulin stimulation there is a second phase of increased glucose uptake that follows after 2 hours. (58).

The glucose uptake in L6 is mediated by facilitated diffusion through plasma membrane transporters. The L6 express GLUT1, GLUT3, and GLUT4 isoforms of the transporters but their expression is dependent on the developmental stage of the cells. The ubiquitous GLUT1 transporter is expressed in both myoblasts and myotubes but the levels decrease during cell differentiation and its main role appears

to be in basal glucose uptake (78,6,58). The GLUT1 proteins also become less glycosylated in myotubes (58) which appears to be important for a GLUT1 structure with higher affinity for glucose and therefore for maintaining high transporter activity (2). The GLUT4 isoform appears to be only expressed to significant levels upon differentiation and is the main contributor to insulin-stimulated glucose uptake (58,6,78). The GLUT3 isoform is expressed in both myoblasts and myotubes with only slightly increased levels in myotubes (6,58). GLUT3 is an isoform found in human fetal skeletal muscle and brain, and only at very low levels in adult muscle. The fact that it is present in L6 myotubes is suggested to be due to the fetal/neonatal characteristic of L6 (6). GLUT1 and GLUT3 levels are concentrated at the plasma membrane while GLUT4 is mostly located in the intracellular stores. Upon insulin stimulation all three isoforms are translocated to the plasma membrane but the largest relative change occurs with the GLUT4 (58). As might be expected from the differences in GLUT expression between myoblasts and myotubes glucose transport activity also differs as the cells develop. Basal glucose transport is highest in myoblasts and decreases as the cells differentiate into myotubes while insulin-stimulated glucose uptake is observed only upon cell alignment and increases as they differentiate to the myotube stage (78).

Culture cells provide the benefit of working with a homogenous group of cells limiting the possibility that variations in response are due to having different types of cells with different responses in each sample as is the case *in vivo* where a certain tissue is composed of different cell types (105). It is also a system which allows the researcher to tightly control the external environment of the cell population thereby

avoiding all the factors seen *in vivo* (e.g. extracellular matrix, blood) that may play a role in the treatment response. Other benefits of cultured cells is that they are more viable than isolated skeletal muscle from biopsies, and the technique is not invasive in nature (105). A particular benefit of using L6 to other models of cultured skeletal muscle is that these cells are well established in expressing the functional GLUT4 transporters and respond well to insulin by causing GLUT4 translocation from an intracellular storage site to the plasma membrane (105,58). The rat L6 skeletal muscle cell line is a well-established *in vitro* model of skeletal muscle which is widely used in studies of insulin signalling and glucose transport.

### 3.4 Preparation of Treatment Compounds

#### Naringenin:

For a 100 mM stock solution: weigh 0.0272g of naringenin and dissolve in 1 ml of ethanol. Vortex and store at 4C. On day of treatment prepare a working stock of 1 mM: Add 10 µl of 100 mM stock per 990µl of 0% FBS media. E.g. Add 100 µl/well of 1 mM working stock for a final [100 µM].

#### Insulin:

Initial solution concentration is 60mM ([100 U/ml]). For a 20mM stock solution: add 100 µl of initial solution to 200µl of 2% FBS media. Store at 4C. For a final [100 nM] add 5µl/well (containing 1 ml media).

#### Nicotinamide:

For a 300mM stock weigh 0.04g of nicotinamide and dissolve in 1092 µl DD water. Store at 4C. For a final [6mM] add 20µl of 300mM stock/ml into wells.

#### STO-609:

Initial solution is 10 mM. For a final [27 $\mu$ M] add 2.7 $\mu$ l/ml into wells.

Ex527:

For a 10 mM stock dissolve 10 mg in 3949 $\mu$ l of DMSO. Protect from light and store at -20C or lower. On day of treatment prepare a 1 mM working stock by adding 10  $\mu$ l of 10 mM stock per 90 $\mu$ l of 0% FBS media. For a final [10  $\mu$ M] add 10  $\mu$ l of 1 mM stock/ml into wells.

### 3.5 Cell Culture and 2-deoxy-D-glucose Uptake

L6 rat skeletal muscle cells were grown for 2 days in  $\alpha$ -MEM containing 5mM glucose, 10% (v/v) FBS, and 1% (v/v) antibiotic-antimycotic solution (100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 250 ng/ml amphotericin B). The cells were then placed in 2% FBS containing media and allowed to differentiate into myotubes. Both myoblasts and myotubes were used depending on the experiments. The cells were serum deprived by placing them in 0% media for 3-5 hours before treatments. The final concentration and the time of incubation for each compound are indicated in each figure (a vehicle control group was used in parallel with the treatment groups). Cells were grown in 6-well plates for western blot experiments and in 12-well plates for glucose uptake experiments. For glucose uptake measurements at the end of treatment, cells were rinsed 3x with HBS at room temperature followed by 2-[<sup>3</sup>H]deoxy-D-glucose uptake measurements by incubating the cells with 300 $\mu$ l of total radioactive buffer in triplicates. Non-specific glucose uptake was assayed using cytochalasin B in a fourth well. The cells were allowed to uptake glucose for 10 minutes at room temperature after which glucose uptake was stopped by washing 3x with cold 0.9% NaCl solution (1 ml/well) followed by lysis by addition of 1 ml/well

of 0.05N NaOH. Prior to lysis cells were examined by microscopic observation. Radioactivity within cells was determined by scintillation counting. Specific glucose uptake (i.e. via glucose transporters) was determined by the following equation where Total is the mean count of three wells:

$$\text{Specific} = \text{Total} - \text{Non Specific}$$

### 3.6 Western blotting

At the end of the treatment period, the cells were rinsed with PBS (1 ml/well) and then lysis buffer (100 µl/well of a 6-well plate) was added, and the lysate was scraped off and stored in eppendorf tubes (1.5 ml). The protein content in each sample was assessed using the BioRad assay. A 3x electrophoresis sample buffer was used to solubilize the lysates. After the addition of the electrophoresis sample buffer the samples were boiled for 5 minutes and stored at  $-20^{\circ}\text{C}$ . Protein samples were loaded into wells of a 10% SDS- polyacrylamide gel and separated by electrophoresis at 120V for ~2 hours. The samples were subsequently transferred from gel to a PVDF membranes at 100V for 1 hour. After transfer the membranes were washed for 5 minutes with 25 ml 1xTBS followed by incubation for 1 hour at room temperature in agitator with 10 ml of 5% (w/v) nonfat dry milk in Tris- buffered saline. Afterwards the membranes were rinsed with 25 ml of TBS/T and then overnight at  $4^{\circ}\text{C}$  with the primary antibody diluted (1:1000) in primary antibody dilution buffer with gentle agitation. The following primary polyclonal antibodies were used: Phospho- Akt (Ser473 and Thr308), Total- Akt, Phospho- AMPK (Thr172), Total-AMPK. The next day the membranes were washed 3x for 5 minutes with 15 ml TBS/T, and the primary antibody was detected with HRP-conjugated secondary antibody diluted in blocking buffer (1:2000) which was incubated with the membrane for at least an hour at room

temperature. This was followed by washing the membranes 3x for 5 minutes with TBS/T and the secondary antibody was detected by ChemiGLO reagent and visualized by FluroChem software via an autodeveloper (ThermoFisher).

### **3.7 Statistical Analysis**

The results are presented as mean  $\pm$  SE of the indicated number of separate experiments using student's paired t-test or analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was assumed at  $P < 0.05$ . Statistical analysis was performed using SPSS v16.0 software for paired t-test, while analysis of variance (ANOVA) was performed using SAS v9.0 software or SPSS v16.0 software.

## CHAPTER 4: RESULTS

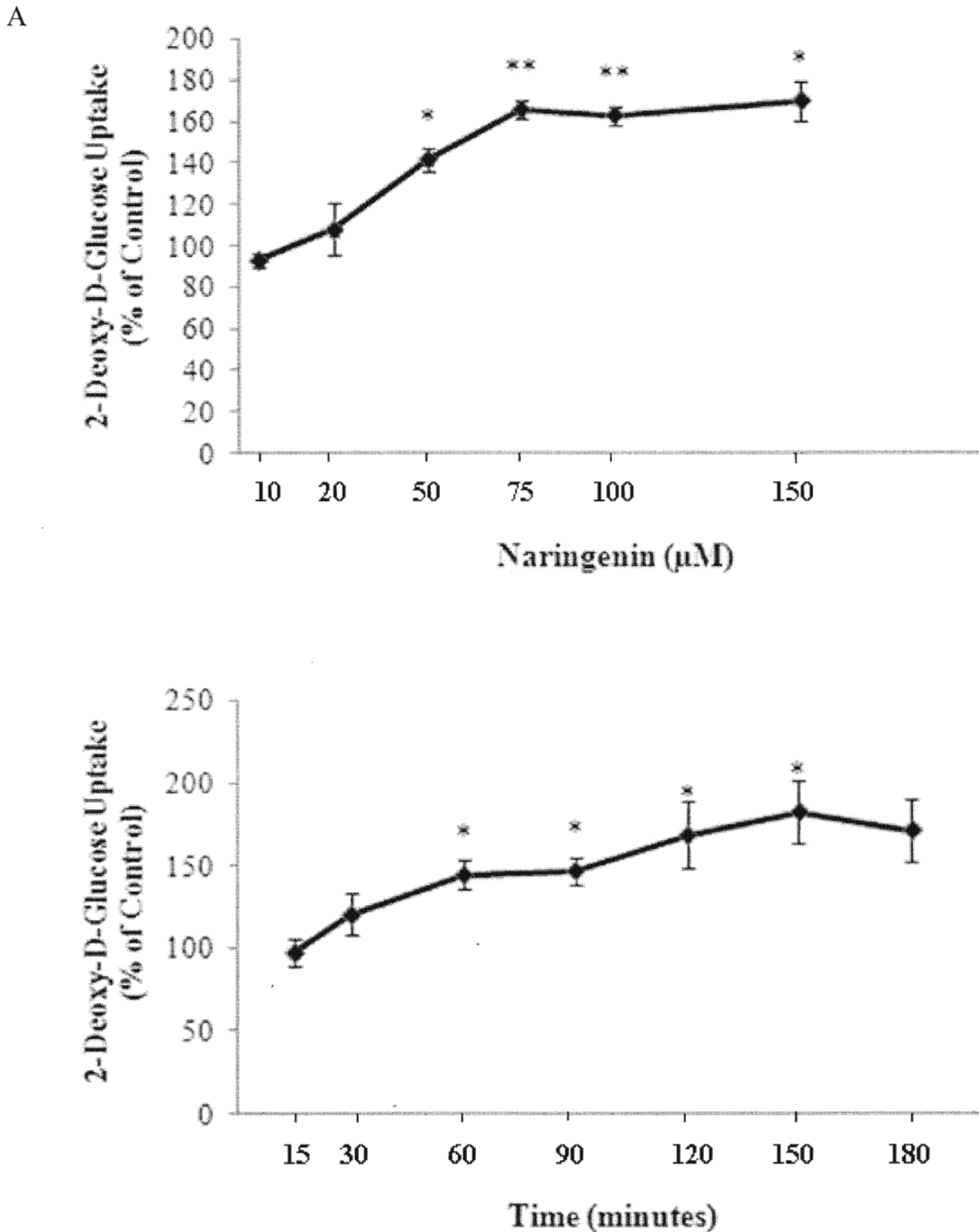
### 4.1 Dose- and time- dependent effects of naringenin on glucose uptake in L6 skeletal muscle.

We investigated whether naringenin may stimulate glucose uptake in L6 skeletal muscle cells. For this purpose L6 myotubes were treated with 10, 20, 50, 75, 100, and 150  $\mu\text{M}$  for 120 minutes followed by glucose uptake measurements (Figure 7A). An incubation time of 120 minutes was initially chosen based on previous studies done in our lab with another polyphenolic compound, resveratrol, which indicated such treatment significantly increased glucose uptake. Naringenin at 5 and 25  $\mu\text{M}$  did not have a significant effect on glucose uptake ( $93\pm3.5\%$  and  $108\pm12.5\%$  of control,  $P>0.05$  respectively). However, at 50  $\mu\text{M}$  naringenin significantly increased glucose uptake ( $141\pm5.5\%$  of untreated control,  $P<0.5$ , Figure 7A). Maximum stimulation was observed at 75  $\mu\text{M}$  ( $165\pm4.9\%$  of control,  $P<0.01$ ). The effect of naringenin reached stability at higher concentrations (100  $\mu\text{M}$  and 150  $\mu\text{M}$ ) and did not further increase glucose uptake.

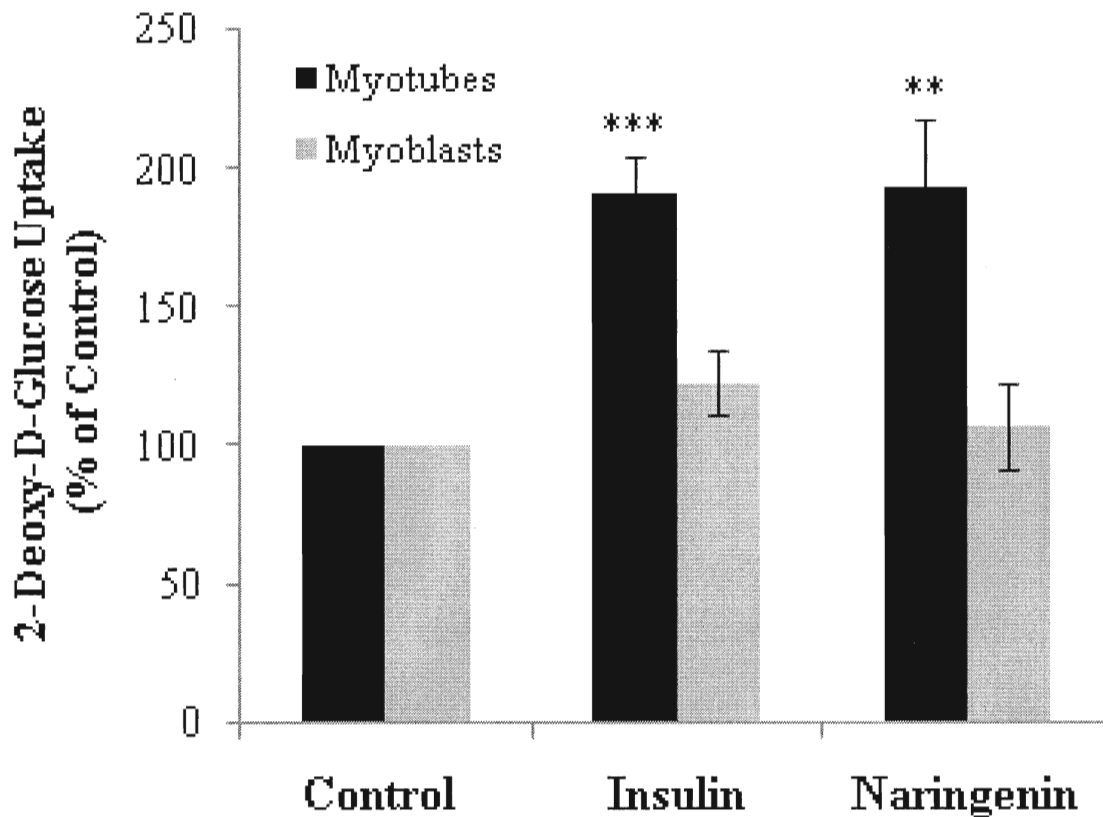
Next, to examine whether the effect of naringenin to increase glucose uptake in myotubes is time-dependent, the cells were incubated with 75  $\mu\text{M}$  of naringenin for 15, 30, 60, 90, 120, 150, and 180 minutes followed by glucose uptake measurements (Figure 7B). Incubation of myotubes with naringenin for 15 and 30 minutes did not result in a significant increase in glucose uptake. However, a significant response was seen at 60 minutes and a maximum response at 120 minutes stimulation ( $144\pm8.7\%$ ,  $P<0.05$ , and  $168\pm20.1\%$ ,  $P<0.05$ , respectively, Figure 7B). Visual microscopic examination showed that cell morphology was not affected by



treatments. Importantly, the effect of naringenin on glucose uptake in myotubes achieved the same level as seen with maximum insulin (100 nM, 30 minutes) stimulation ( $192.8 \pm 24\%$ ,  $P < 0.01$  and  $190.1 \pm 13.3\%$ ,  $P < 0.001$ , respectively, Figure 8). These results suggest that naringenin significantly stimulates glucose uptake in a dose- and time-dependent manner that is independent of insulin. Insulin's effect to increase glucose uptake in muscle is largely mediated by GLUT4 transporters, therefore to investigate whether a similar mechanism may be involved in the naringenin-stimulated increase in glucose uptake we also examined whether naringenin increases glucose uptake in L6 myoblasts. As the L6 cell line differentiates from myoblast to myotubes they differ also in the degree of GLUT1 and GLUT4 transporter expression. L6 myoblasts are known not to respond to insulin due to low GLUT4 expression. Similar to insulin, treatment with naringenin did not significantly increase glucose uptake in L6 myoblasts ( $122.3 \pm 11.8\%$  and  $106.3 \pm 15.2\%$ , respectively,  $P > 0.05$ , Figure 8), indicating that the effect of naringenin to increase glucose uptake in myotubes may be due to modulation of GLUT4 translocation and/or activity.



**Figure 7:** (A) Effects of naringenin on 2DG uptake by skeletal muscle cells: **Dose Response**. L6 myotubes were incubated with the indicated concentrations of naringenin for 120 minutes at 37°C. (B) Effects of naringenin on 2DG uptake by skeletal muscle cells: **Time Course**. L6 myotubes were incubated with 75μM naringenin for the indicated time periods at 37°C. At end of treatments 2DG uptake was performed. Results are the mean  $\pm$  SE of 2-5 experiments expressed as percent of control. \*P< 0.05, \*\* P<0.01, \*\*\*P<0.001.

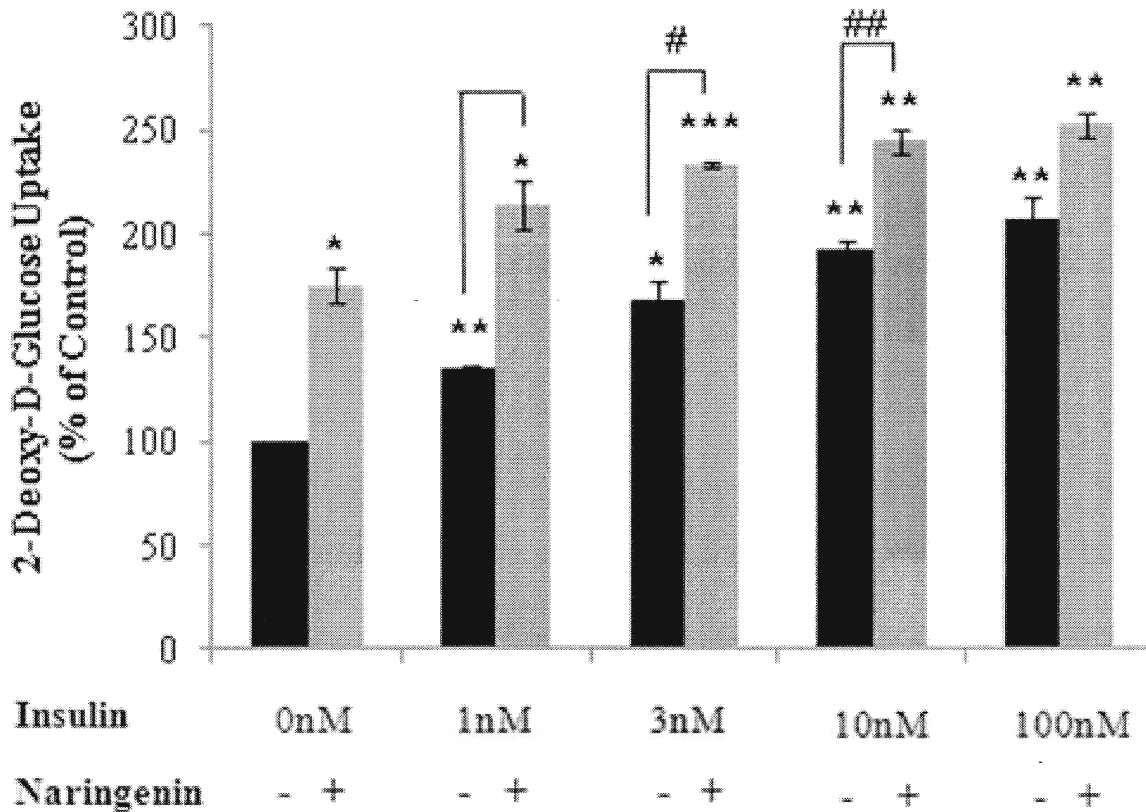


**Figure 8:** Effects of naringenin and insulin on 2DG uptake by myotubes (dark bars) and myoblast (light bars). L6 myotubes were incubated with or without 100 nM insulin (I) for 30 minutes or 75  $\mu$ M concentrations of naringenin for 2 hours at 37°C. At end of treatments 2DG uptake was performed. Results are the mean  $\pm$  SE of 10 experiments (dark bars) and  $\pm$  SE of 3 experiments (light bars) expressed as percent of control. \* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\* $P < 0.001$ .

#### 4.2 Effects of naringenin on insulin-stimulated glucose uptake.

In addition to naringenin's effect alone we also investigated whether naringenin would have an additive effect on glucose uptake when combined with acute insulin stimulation. To this effect myotubes were treated with or without 100  $\mu$ M of naringenin (120 minutes total incubation) and with or without varying concentrations of insulin for the last 30 minutes. Insulin concentrations included 1 nM, 3 nM, 10 nM, and 100 nM. Glucose uptake measurements were performed following treatments.

Insulin dose-dependently increased glucose uptake ( $134\pm1.8\%$ ,  $167\pm9.8\%$ ,  $192\pm4.9\%$ ,  $207\pm10.6\%$  of control, in order of increasing concentration) (Figure 9). Pre-treatment with naringenin significantly increased glucose uptake for the sub-maximal insulin treatment groups (1 nM, 3 nM, 10 nM) as compared to treatment with insulin alone ( $214\pm11.6\%$ ,  $233\pm0.9\%$ ,  $244\pm6.4\%$  of control, in order of increasing insulin concentration) (Figure 9). With maximal levels of insulin naringenin's capacity to further increase glucose uptake tended to be additive but did not reach significantly increased levels above insulin treatment alone ( $252\pm5.6\%$  vs.  $207\pm10.6\%$  of control, respectively,  $p=0.093$ ) (Figure 9). The results indicate that naringenin has an additive effect on the insulin-stimulated glucose uptake only at sub-maximal concentrations of insulin.

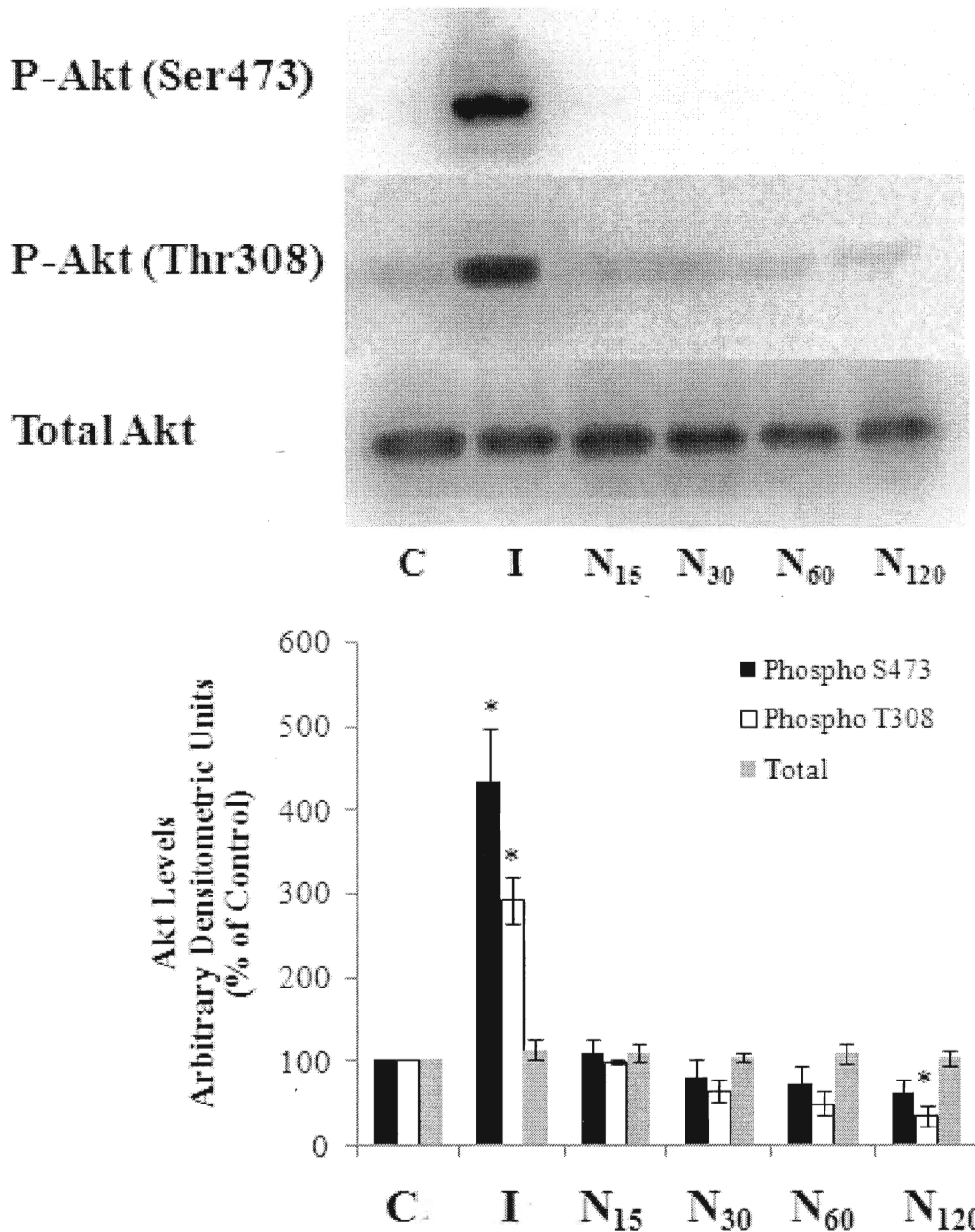


**Figure 9:** Naringenin has an additive effect on the insulin stimulated glucose uptake in myotubes. L6 myotubes were serum deprived for 3-5 hours and then pre-treated with 100  $\mu$ M naringenin for 90 minutes followed by treatment with or without various concentrations of insulin for 30 minutes at 37°C. At end of treatments 2DG uptake was performed. Results are the mean  $\pm$  SE of 3 experiments expressed as percent of control.

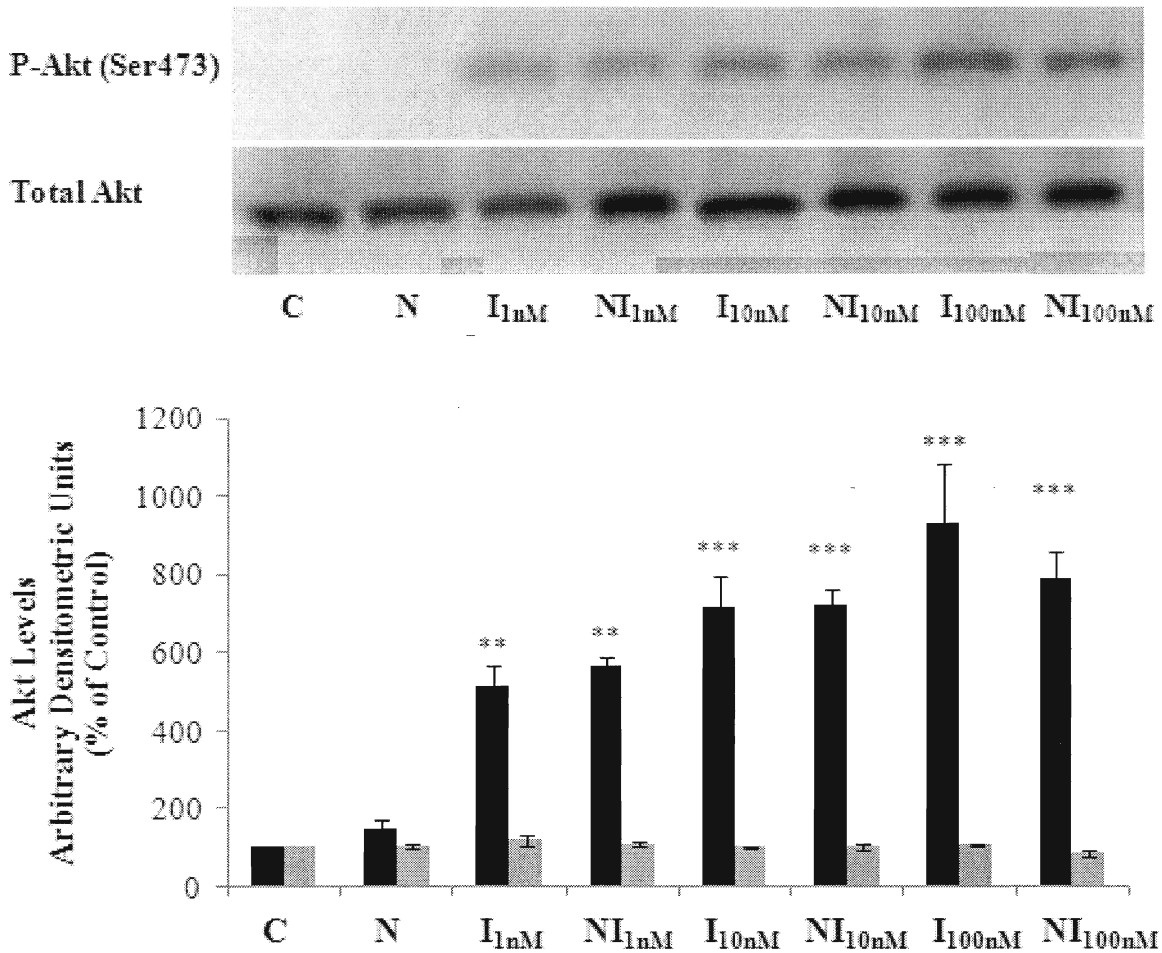
### 4.3 Effects of naringenin on Akt phosphorylation at Ser473 and Thr308.

In order to elucidate the mechanism of the naringenin-mediated increase in glucose uptake, given the similarity of naringenin's effect with that of insulin, we next examined the effect of naringenin on Akt, a molecule involved in the insulin signalling cascade leading to increased glucose uptake in myotubes. The activity of Akt is regulated by phosphorylation and the phosphorylation at two of its residues - Ser473 and Thr308 - is required for its activation within this pathway (55,114). The effect of naringenin on the phosphorylation of these two residues was examined using phospho-specific antibodies in western blot analysis. As expected, insulin treatment (100 nM for 15 minutes) strongly stimulated phosphorylation of both Ser473 and Thr308 Akt residues ( $367 \pm 12.5\%$ ,  $P < 0.05$ , and  $262.5 \pm 7.5\%$ ,  $P < 0.05$ , Figure 10A). However, treatment with 100  $\mu$ M of naringenin for 15, 30, 60, or 120 minutes did not increase the phosphorylation of either of the Akt residues (Figure 10A).

Although naringenin did not affect basal Akt phosphorylation the possibility existed that the observed additive effect of naringenin on insulin-stimulated glucose uptake may be modulated by potentiating the insulin-stimulated Akt phosphorylation. To examine this possibility L6 myotubes were treated with or without 100  $\mu$ M of naringenin, followed by treatment with or without different concentrations of insulin (1 nM, 10 nM, 100 nM). As expected, insulin treatment increased Akt phosphorylation at Ser473 (Figure 10B) and tended to increase Akt phosphorylation at Thr308 (Figure 10C). However, pre-treatment with naringenin did not significantly affect the insulin-stimulated Akt phosphorylation (Figure 10B,C). These results suggest that the effect of naringenin on glucose uptake is Akt-independent.



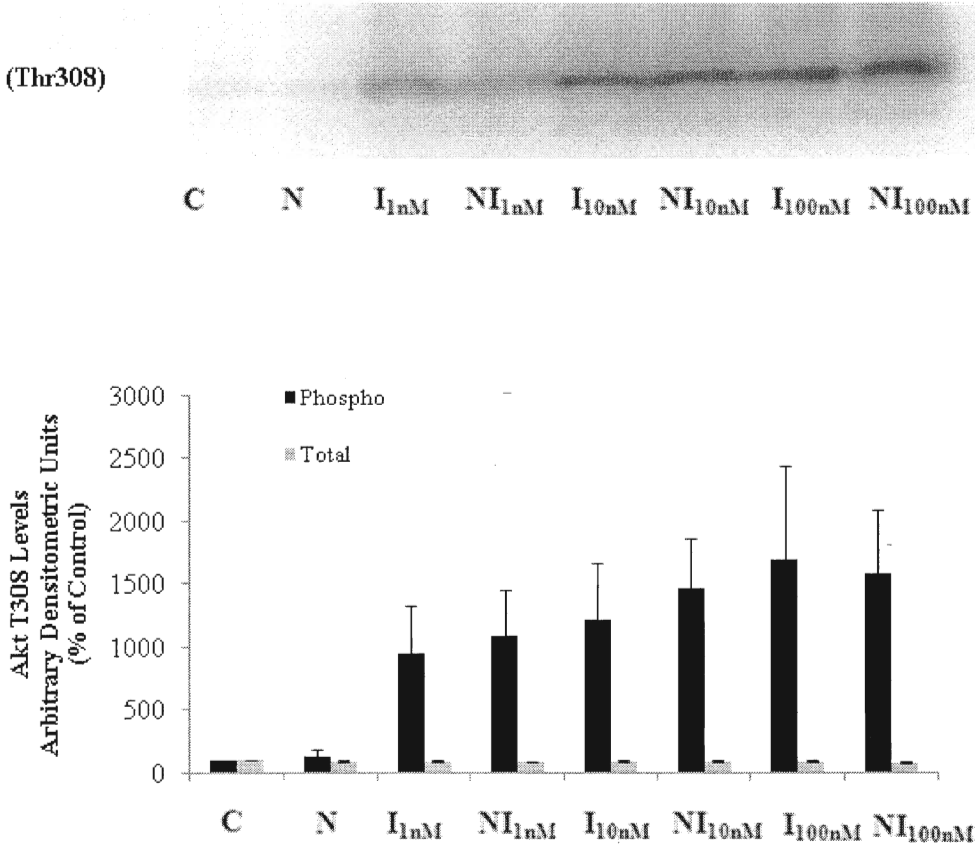
**Figure 10: (A)** Time-course: Effects of naringenin on Akt phosphorylation/activation in skeletal muscle. L6 myotubes were serum deprived for 3-5 hours and then incubated with 100 nM insulin and/or 100  $\mu$ M naringenin for the indicated time in minutes. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-Akt (Ser473 and Thr308) and total-Akt. Results are the mean  $\pm$  SE of 3-5 experiments expressed as percent of control. \* $p < 0.05$  compared to untreated control.



**Figure 10:** (B) Effects of naringenin on insulin-stimulated Akt phosphorylation at Ser473 in skeletal muscle. L6 myotubes were serum deprived for 3-5 hours and then pre-treated with 100  $\mu$ M naringenin for 30 minutes followed by treatment with or without various concentrations of insulin for 15 minutes. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-Akt and total-Akt. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$  compared to untreated control, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  compared to insulin treatment alone.



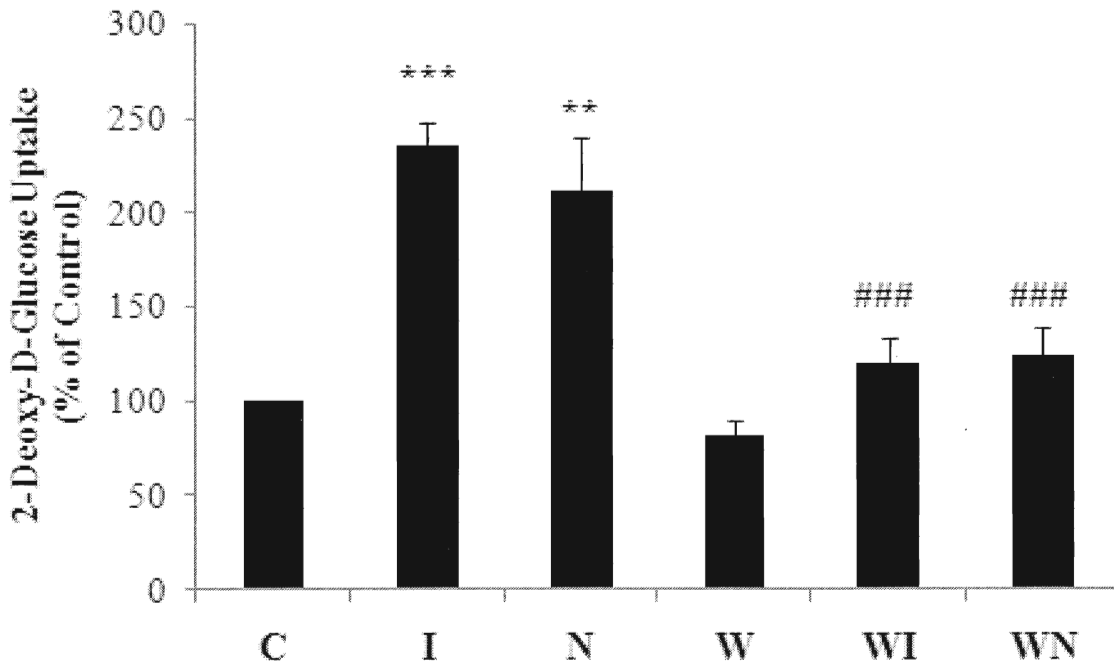
P-Akt (Thr308)



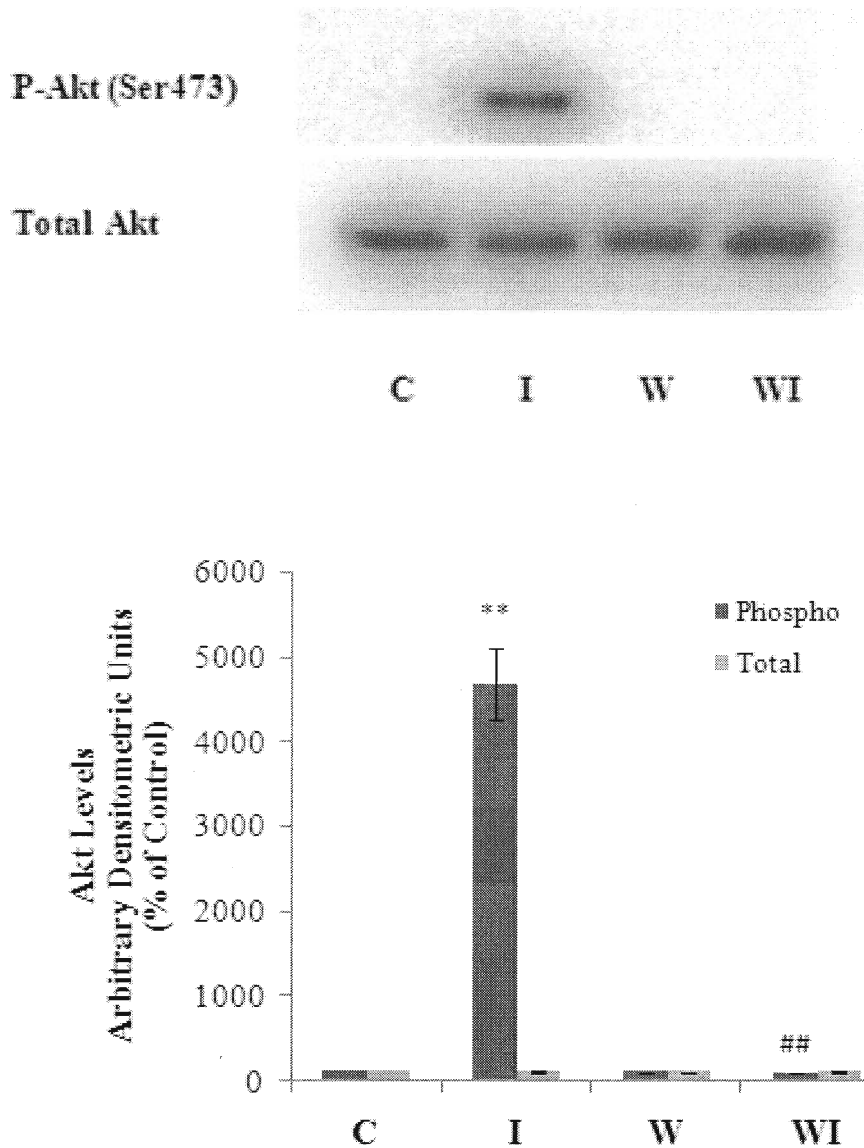
**Figure 10:** (C) Effects of naringenin on insulin-stimulated Akt phosphorylation at Thr308 in skeletal muscle. L6 myotubes were serum deprived for 3-5 hours and then pre-treated with 100  $\mu$ M naringenin for 30 minutes followed by treatment with or without various concentrations of insulin for 15 minutes. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-Akt and total-Akt.

#### 4.4 Effects of PI3K inhibition on naringenin-stimulated glucose uptake.

PI3K has been established as a key molecule involved in the insulin signalling cascade that leads to glucose uptake in skeletal muscle and its activity is required in this action (15,1,55,91). Therefore we investigated whether PI3K may also be a mediator of naringenin-stimulated glucose uptake by using a PI3K inhibitor, wortmannin. Myotubes were pre-treated with 1  $\mu$ M of wortmannin for 15 minutes followed by treatment with or without 100  $\mu$ M naringenin for 120 minutes or 100 nM insulin for 30 minutes. After treatments glucose uptake measurements were performed. The insulin- and naringenin-stimulated increase in glucose uptake ( $235\pm12.4\%$  and  $212\pm27.1\%$ ,  $p<0.001$  and  $p<0.01$ , respectively) was inhibited with wortmannin pre-treatment ( $120\pm13\%$  and  $123\pm15.7\%$  of control,  $p<0.001$  compared to insulin or naringenin alone, respectively) (Figure 11A). These results suggest that PI3K is involved in the regulation of glucose uptake by naringenin. To confirm the effectiveness of wortmannin to inhibit PI3K we examined its effect on phosphorylation of Akt of insulin treated cells, as Akt is a molecule downstream of PI3K in the insulin-signalling cascade. Treatment of myotubes with 100 nM insulin for 15 minutes significantly increased Akt phosphorylation ( $4679\pm434\%$  of control,  $p<0.05$ ) and pre-treatment with 1  $\mu$ M of wortmannin for 15 minutes significantly inhibited this phosphorylation ( $83\pm5.5\%$  of control,  $p<0.05$ ) which would indicate that wortmannin had successfully inhibited insulin signalling (Figure 11B).



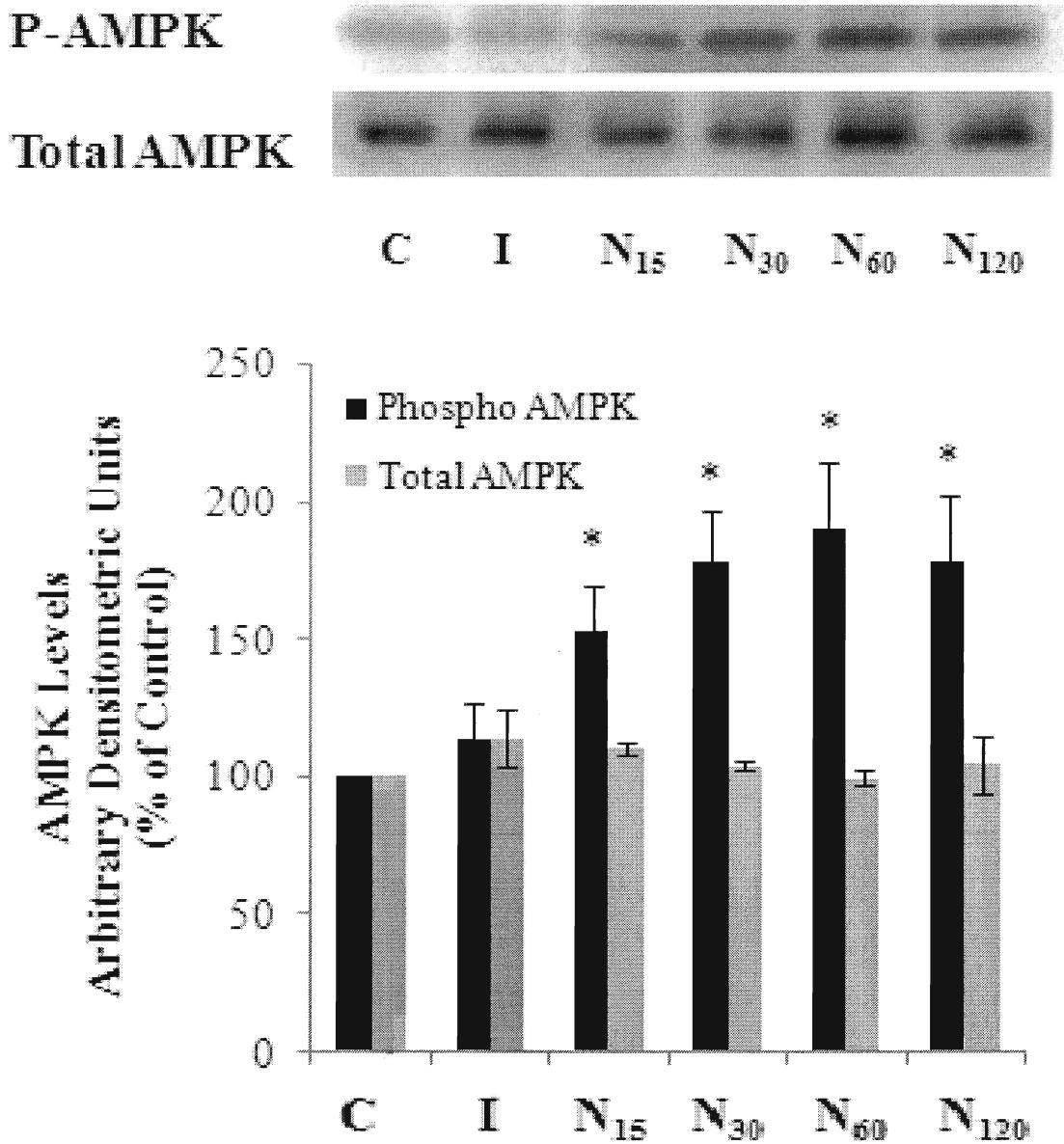
**Figure 11:** (A) Effect of PI3K inhibition on insulin- and naringenin-stimulated glucose uptake: L6 myotubes were serum deprived for 3-5 hours and then incubated with 1  $\mu$ M of wortmannin for 15 minutes followed by treatment with 100 nM insulin for 30 min or 100  $\mu$ M naringenin for 2 hours. At end of treatments 2DG uptake was performed. Results are the mean  $\pm$  SE of 8 experiments expressed as percent of control. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$  compared to untreated control, # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  compared to insulin or naringenin treatment alone.



**Figure 11:** (B) Effects of wortmannin on insulin-stimulated Akt phosphorylation: L6 myotubes were serum deprived for 3-5 hours and then pre-treated with 1  $\mu$ M of wortmannin followed by treatment with 100 nM insulin for 15 minutes. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-Akt and total Akt. \*\*  $p < 0.01$  compared to untreated control, ##  $p < 0.01$  compared to insulin treatment alone.

#### 4.5 Effects of naringenin on AMPK phosphorylation.

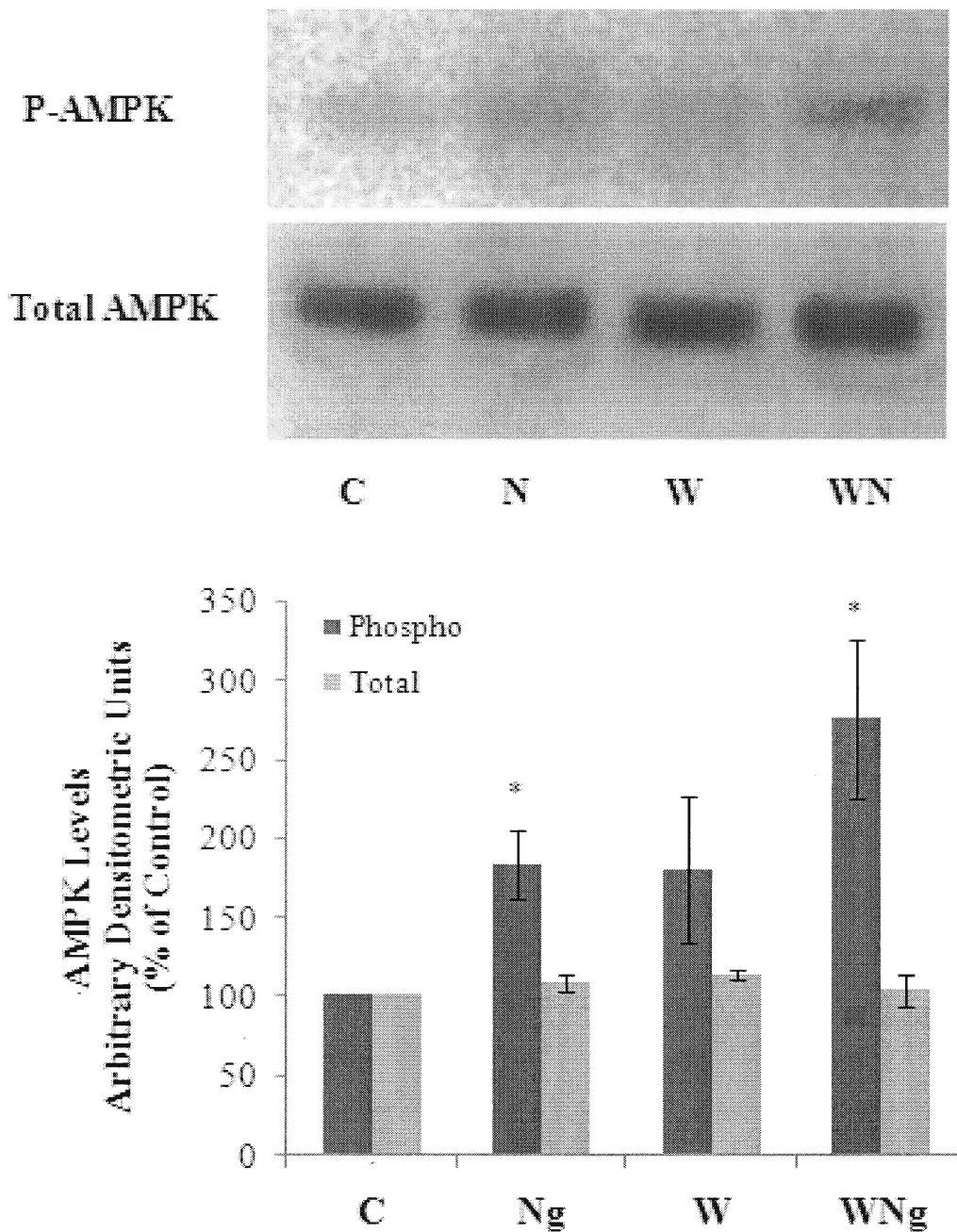
AMPK in skeletal muscle has been shown to be another mechanism stimulating glucose uptake. It is activated by exercise/contraction and numerous compounds including metformin, thiazolidineones, and the polyphenol resveratrol (13,28,35,84,110,126). Since resveratrol, which has structural similarities with naringenin, has been reported to activate AMPK the effect of naringenin on AMPK phosphorylation was also examined as a potential mechanism for its glucose uptake stimulating effect. Myotubes were treated with or without 100  $\mu$ M naringenin for 15, 30, 60, and 120 minutes. Western blot analysis showed that naringenin treatment increased AMPK phosphorylation (Figure 12). A significant increase in AMPK phosphorylation was observed within 15 minutes of naringenin treatment ( $153.2 \pm 16.3\%$ ,  $P < 0.05$ ) and peak levels were observed within 60 minutes ( $190.4 \pm 23.9\%$ ,  $P < 0.05$ ) and remained elevated at 120 minutes ( $178 \pm 23.4\%$ ,  $P < 0.05$ ). As expected, insulin did not significantly increase AMPK phosphorylation ( $113 \pm 13\%$ ,  $P > 0.05$ , Figure 12).



**Figure 12:** Time-course: Effects of naringenin on AMPK phosphorylation/activation in skeletal muscle. L6 myotubes were serum deprived for 3-5 hours and then incubated with 100 nM insulin or 100  $\mu$ M naringenin for the indicated time (minutes). After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-AMPK (Thr172) and total-AMPK. Results are the mean  $\pm$  SE of 5 experiments expressed as percent of control. \* $p < 0.05$  compared to untreated control.

#### **4.6 Effects of PI3K inhibition on naringenin-stimulated AMPK phosphorylation.**

Because activation of AMPK is known to lead to increased glucose uptake in skeletal muscle and so far the results indicate that naringenin activates AMPK and that PI3K appears to be involved in the naringenin-stimulated glucose uptake we were interested in examining whether PI3K inhibition would inhibit naringenin-stimulated AMPK phosphorylation. Myotubes were pre-treated with or without 1  $\mu$ M of wortmannin for 15 minutes followed by treatment with or without 100  $\mu$ M naringenin for 60 minutes. Western blot analysis revealed that naringenin treatment stimulated AMPK phosphorylation ( $183 \pm 21.59\%$  of control) and pre-treatment with wortmannin did not inhibit the naringenin-mediated AMPK phosphorylation but rather tended to have an additive effect ( $275 \pm 49.7\%$ ) (Figure 13). These results suggest that PI3K is not an upstream mediator of naringenin-induced AMPK phosphorylation.



**Figure 13:** Effects of PI3K inhibition on naringenin-mediated AMPK phosphorylation. L6 myotubes were serum deprived for 3-5 hours and then pre-treated with 1  $\mu$ M of wortmannin followed by treatment with 100  $\mu$ M naringenin for 60 minutes. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-AMPK (Thr172) and total-AMPK. Results are the mean  $\pm$  SE of 4 experiments expressed as percent of control. \* $p$  < 0.05 compared to untreated control.



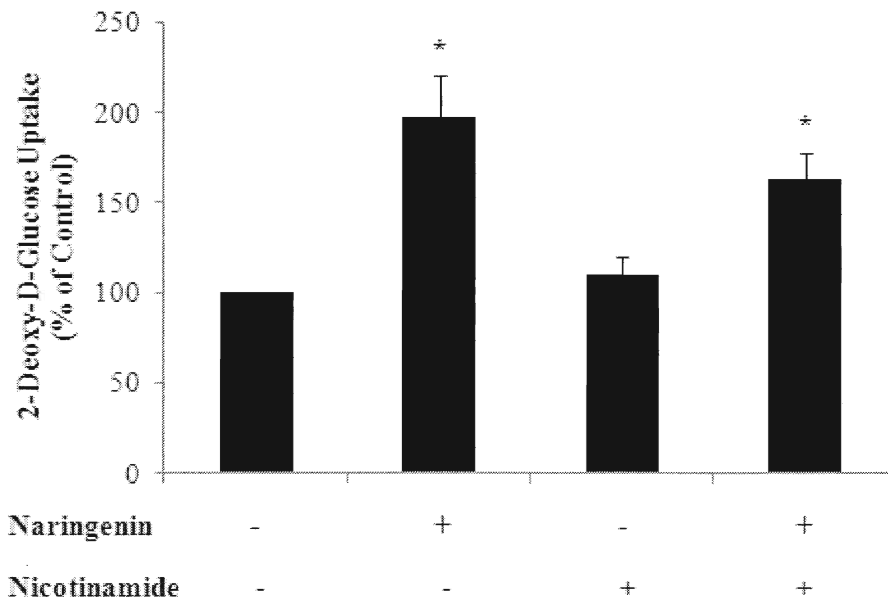
#### **4.7 Effects of SIRT1 inhibition on naringenin-stimulated glucose uptake and on phosphorylation of AMPK and LKB1.**

Recent reports indicate that activation of the protein deacetylase SIRT1 may lead to AMPK phosphorylation by increasing phosphorylation/activation of LKB1, an upstream kinase of AMPK (66). Additionally SIRT1 has recently been implicated in the mechanism of the structurally similar resveratrol to increase AMPK phosphorylation (46). We therefore hypothesized that SIRT1 may mediate the effect of naringenin to stimulate AMPK phosphorylation and thereby mediate glucose uptake in skeletal muscle cells. Furthermore, SIRT1 may lead to increased activity of LKB1 which may mediate its effect on AMPK. To test this hypothesis we conducted experiments using SIRT1 inhibitors, nicotinamide and Ex527. The inhibitory mode of action of nicotinamide is via product inhibition. The deacetylation activity of SIRT1 requires bond cleavage between the ADP-ribose and nicotinamide portions of NAD. This releases nicotinamide which can rebind to reform NAD thereby reversing the deacetylation reaction. Nicotinamide accumulation favours reformation of NAD thereby inhibiting SIRT1 (87). Ex 527 is a cell permeable, potent, and selective inhibitor of SIRT1 that appears to work by binding to the enzyme after the release of nicotinamide and prevent the release of the deacetylated substrate (87).

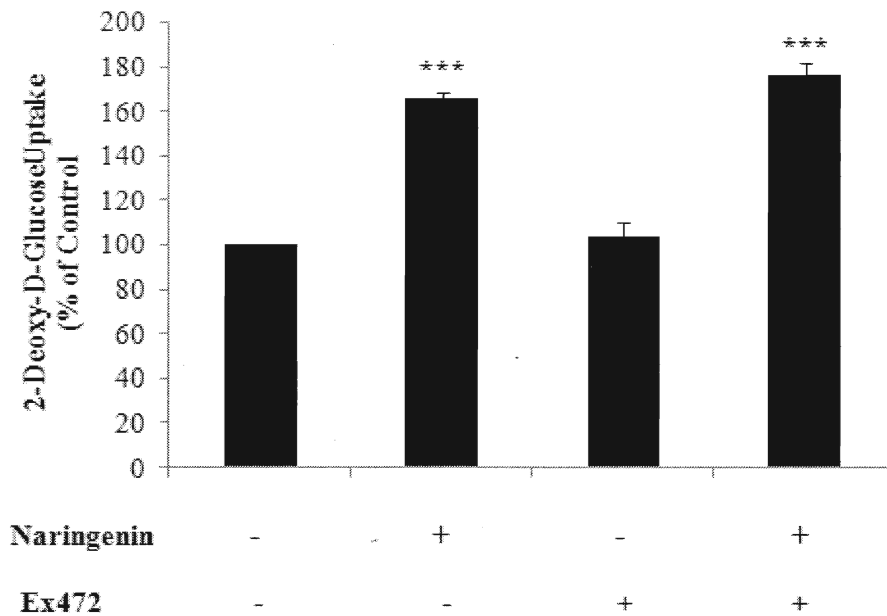
First, we examined the effect of SIRT1 inhibition on naringenin-stimulated glucose uptake. Myotubes were pre-treated with either 6 mM nicotinamide for 4 hours or 10  $\mu$ M Ex527 for 12 hours followed by treatment with or without 100  $\mu$ M naringenin for 2 hours. As expected, naringenin increased glucose uptake ( $182 \pm 12.2\%$  of control,  $p < 0.001$ , Figure 14 A,B). However, neither nicotinamide or

Ex527 inhibited glucose uptake by naringenin ( $162 \pm 15.2\%$  and  $176 \pm 5.4\%$  of control, respectively, Figure 14A,B) suggesting that SIRT1 is not involved in the mechanism of naringenin on glucose uptake. Although SIRT1 inhibitors had no effect on naringenin-mediated glucose uptake the possibility still existed that SIRT1 may be involved in mediating naringenin's effect on AMPK. If so this would rule out AMPK as a mediator of naringenin's effect on glucose uptake. Therefore, myotubes were incubated with either 6mM nicotinamide for 4hours or 10  $\mu$ M Ex527 for 12 hours followed by treatment with 100  $\mu$ M naringenin for 60 minutes. Western blot analysis showed that naringenin increased AMPK phosphorylation ( $191 \pm 23.3\%$  of control and  $697 \pm 323.6\%$  of control, Figure 14C and D, respectively). SIRT1 inhibition by either nicotinamide or Ex527 did not inhibit AMPK phosphorylation, tending rather to increase AMPK phosphorylation. As shown in Figure 14D naringenin treatment had no effect on the phosphorylation of LKB1 nor did SIRT1 inhibition by Ex527.

A

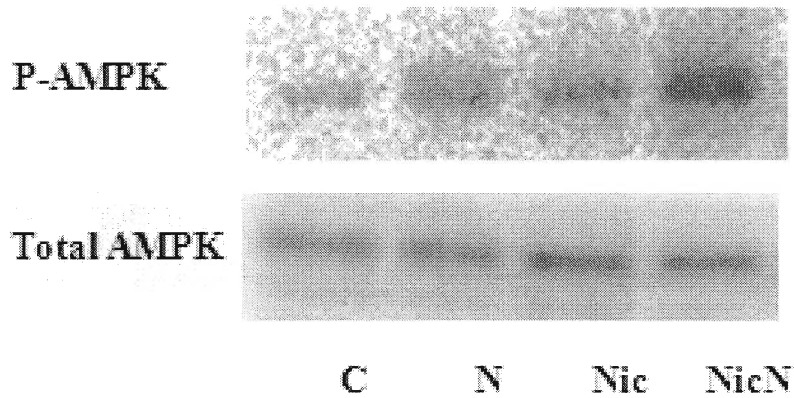


B

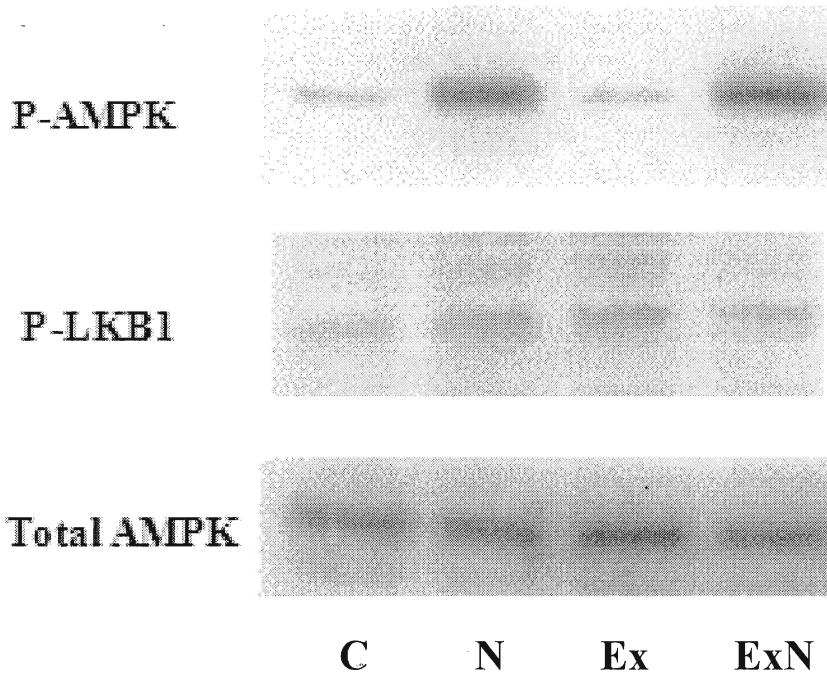


**Figure 14:** (A,B) Effects of SIRT1 inhibitors on naringenin mediated glucose uptake. (A) L6 myotubes were serum deprived and pre-treated with 6mM nicotinamide for 4 hours before incubating with 100  $\mu$ M naringenin for 2 hours. (B) L6 myotubes were serum deprived and pre-treated with 10  $\mu$ M Ex527 for 12 hours before incubating with 100  $\mu$ M naringenin for 2h. At end of treatments 2DG uptake was performed. Results are the mean  $\pm$  SE of 5 experiments expressed as percent of control. \* $p$  < 0.05, \*\*  $p$  < 0.01, \*\*\* $p$  < 0.001 compared to untreated control.

C



D



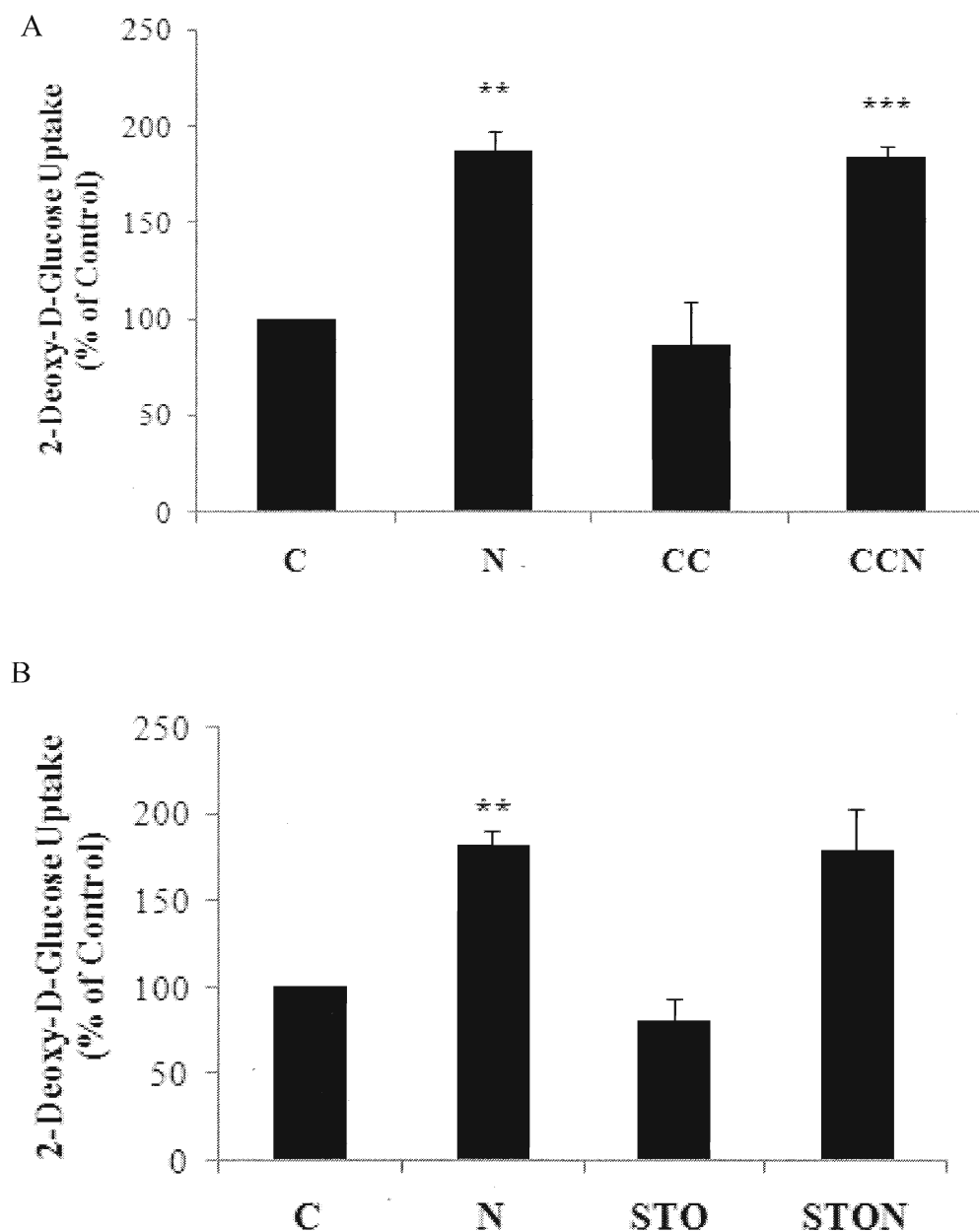
**Figure 14:** (C) L6 myotubes were serum deprived and pre-treated with 6 mM nicotinamide for 4 hours before incubating with 100  $\mu$ M naringenin for 1 hour. (D) L6 myotubes were serum deprived and pre-treated with 10  $\mu$ M Ex527 for 12 hours before incubating with 100  $\mu$ M naringenin for 1 hour. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-AMPK (Thr172), total-AMPK, and phospho-LKB1. Representative blots of 3 experiments.

#### 4.8 Effects of AMPK inhibitors on naringenin mediated glucose uptake.

Activation of AMPK has been shown to lead to increased glucose uptake in several cell types and this molecule has been shown to be involved in the therapeutic effects of exercise, contraction, and anti-diabetic agents on metabolism. Our data indicates that naringenin stimulates AMPK phosphorylation therefore we hypothesized that here too AMPK activity may mediate the increase in glucose uptake. Due to its nature as an energy sensor and regulator the AMPK molecule appears to have multiple ways of being activated. SIRT1 is a newly identified potential activator of AMPK but it is only one of the possible ways AMPK may be stimulated. In the case of naringenin our data would suggest that SIRT1 is not involved; here is one example in which the two polyphenols, naringenin and resveratrol, seem to differ. To further test our hypothesis we sought other ways to pharmacologically inhibit AMPK activity and test the effects on naringenin-stimulated glucose uptake. Two other ways that AMPK is activated is by a cellular increase in AMP: ATP ratio and by the upstream activator of AMPK calmodulin-dependent protein kinase kinase (CaMKK) (35,31,13). Activation of CaMKK is not mediated by increasing the AMP: ATP ratio but rather by a rise in calcium (25,31). We therefore used compound C (CC), an ATP-competitive AMPK inhibitor, and STO-609, an inhibitor of CaMKK, to examine their effect on naringenin-stimulated glucose uptake. STO-609 is a cell permeable and selective inhibitor of CaMKK  $\alpha$  and  $\beta$  isoforms that is reported to work by competitive inhibition of ATP (115).

Myotubes were pre-treated with 25  $\mu$ M CC for 30 minutes followed by treatment with or without 100  $\mu$ M naringenin for 2 hours (Figure 15A). As expected

naringenin increased glucose uptake ( $187 \pm 10.5\%$  of control,  $p < 0.01$ , Figure 15A), however pre-treatment with CC did not inhibit this glucose uptake. Myotubes were also pre-treated with  $27 \mu\text{M}$  STO-609 for 60 minutes followed by treatment with  $100 \mu\text{M}$  naringenin for 2 hours (Figure 15B). Again naringenin significantly increased glucose uptake ( $184 \pm 5.6\%$  of control,  $p < 0.01$ ). As was the case with CC, STO-609 pre-treatment did not inhibit the naringenin-stimulated glucose uptake, tending to retain similar levels as naringenin treatment alone ( $180 \pm 23.5\%$  of control,  $p = 0.077$ , Figure 15B). Although these results do not rule out AMPK's involvement in the naringenin effect on glucose uptake, because there could be other ways of AMPK activation, they do bring its involvement into doubt. The results suggest that either AMPK is not involved or that naringenin activates AMPK via a mechanism that does not involve SIRT1, CaMKK, or changes in AMP:ATP ratio.

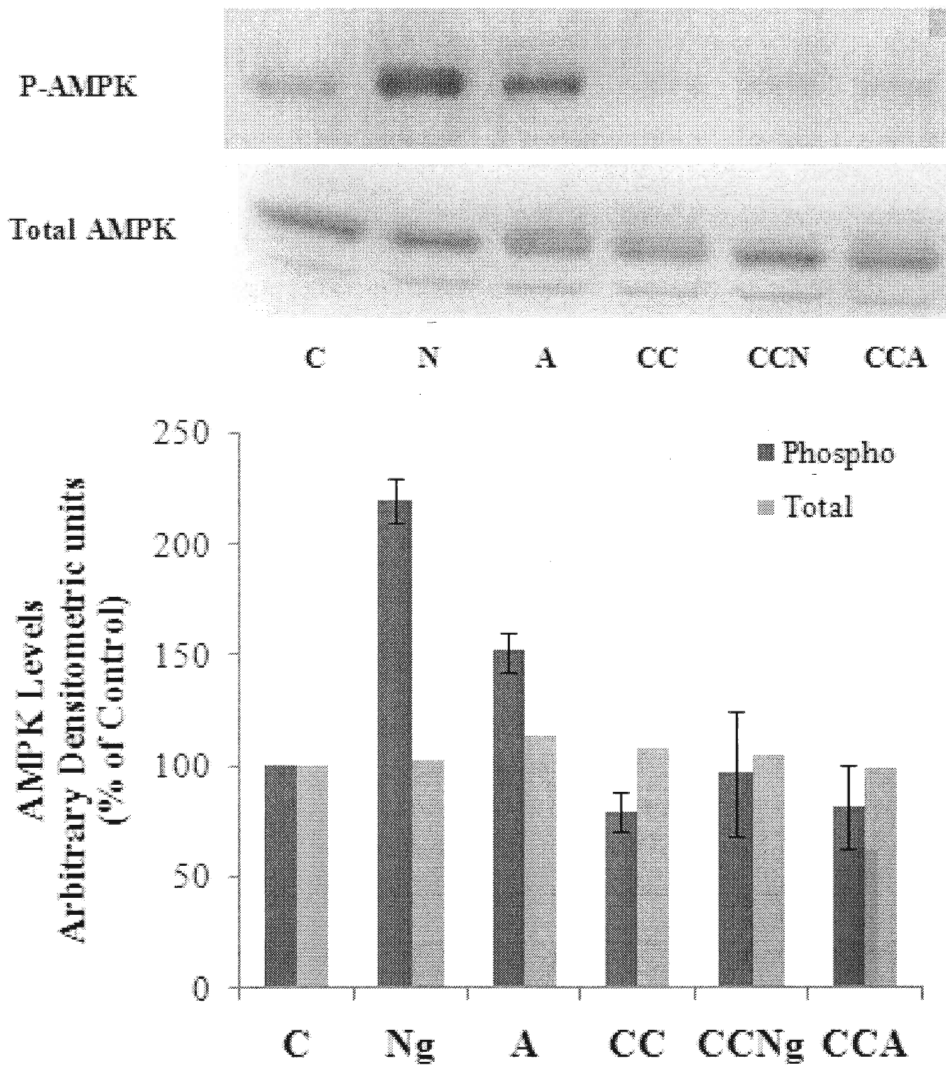


**Figure 15:** Effects of AMPK inhibitors on naringenin mediated glucose uptake. (A) L6 myotubes were serum deprived and pre-treated with 25  $\mu$ M CC for 30 min followed by treatment with or without 100  $\mu$ M naringenin for 2h. At end of treatments [ $^3$ H] 2-deoxy-D glucose uptake was performed. Results are the mean  $\pm$  SE of 4 experiments expressed as percent of control. (B) L6 myotubes were serum deprived and pre-treated with 27  $\mu$ M STO-609 for 60 min followed by treatment with 100  $\mu$ M naringenin for 2h. At end of treatments 2DG uptake was performed. Results are the mean  $\pm$  SE of 3 experiments expressed as percent of control. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 compared to untreated control.

#### **4.9 Effects of Compound C on naringenin-stimulated AMPK phosphorylation.**

From previous results further elucidation was necessary of the role of AMPK in the naringenin-stimulated glucose uptake. We sought to determine whether the phosphorylation of AMPK by naringenin would be inhibited by CC. This would shed light also on whether the phosphorylation of AMPK in this case may involve an increase in AMP:ATP ratio. Myotubes were pre-treated with 25  $\mu$ M CC for 30 minutes followed by treatment with 100  $\mu$ M naringenin for 60 minutes or 2 mM AICAR for 2 hours. AICAR (5-Aminoimidazole-4-carboxamide ribonucleoside) is an analog of adenosine which, when taken up into myotubes is converted to 5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5'-monophosphate (ZMP). ZMP then activates AMPK which has been shown to be inhibited by CC and therefore served as a positive control of CC inhibition in the experiment. As shown in Figure 16, both naringenin and AICAR stimulated AMPK phosphorylation and the action of both was inhibited by CC pre-treatment. This suggests that naringenin may have stimulated AMPK phosphorylation by causing an increasing in AMP: ATP ratio as well as suggesting that the stimulation of AMPK is not required for naringenin-induced increase in glucose uptake.





**Figure 16:** Effects of CC on naringenin- and AICAR-stimulated AMPK phosphorylation. L6 myotubes were serum deprived and pre-treated with 25  $\mu$ M CC for 30 minutes followed by treatment with or without 100  $\mu$ M naringenin for 60 minutes. After treatment total cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with specific anti-bodies that recognize phospho-AMPK (Thr172), and total-AMPK.

## CHAPTER 5: DISCUSSION

### 5.1 Naringenin significantly increases glucose uptake.

Diabetes mellitus is a disease affecting the endocrine system which makes it a complex disease to treat. The dysfunction, inadequate insulin action to promote metabolic effects, has consequences on numerous tissues and functions. This is of course because the characteristics of the endocrine form of cellular communication are that it tends to be spread throughout the body, and affect many cells and tissues in diverse ways. Insulin is not an exception: it is involved in regulating metabolism and so acts on different target tissues and cell types, impacting glucose, lipid, and protein metabolism in an effort to coordinate the body to adopt the proper metabolism as needed (5,55,63,99,102). It is no wonder then that until the root dysfunction(s) can be treated, management of this disease is difficult. For type 1 diabetes, where the dysfunction is insufficient insulin, which can be corrected by administering insulin, the difficulty in management of the disease is in appropriately timing the treatment to approximate the normal physiological response that the body would have to the ever changing supply and demand of energy (110,123). In type 2 diabetes, where the dysfunction is an inadequate response to insulin, this difficulty is compounded in that the treatments available are in themselves approximations of insulin. There is no treatment that can do all that insulin would do in the body, in a healthy state. Yet another problem is that the dysfunction in type 2 diabetes is heterogeneous and therefore different people may need a different treatment regiment to address their particular points of deregulation (19,1,63,77,82,83,123). Therefore, the study of novel compounds that may mimic the effects of insulin on target tissues remains an

important area of research both for potentially finding more treatment options, as well as for increasing our knowledge of metabolic regulation in health and disease.

One of the points of regulation that is disrupted in type 2 diabetes is peripheral glucose uptake. Skeletal muscle tissue is one of the targets of insulin and it is responsible for a major portion, ~80%, of glucose disposal in the post-prandial state (85). For this reason interventions that can improve glucose uptake in this tissue are of major interest. Naringenin has been reported to have antioxidant, antiproliferative, anticancer, and anti-inflammatory properties (1,22,92). Effects on glucose and lipid metabolism have also been reported. *In vitro* studies have shown that naringenin has an insulin-like effect to decrease basal oleate-stimulated apolipoprotein B secretion in hepatocytes (7,8,9). In *in vivo* studies naringenin exhibited a hypoglycaemic effect in male wistar rats, and in streptozotocin-induced diabetic rats intraperitoneal administration of naringenin significantly decreased plasma glucose, triglycerides and total cholesterol levels (18,17,70). Furthermore naringenin has structural similarities with the extensively studied resveratrol which reportedly has antidiabetic properties both *in vitro* and *in vivo* (11,110,3.1,4,65,93,112.1). Therefore we examined the effects of naringenin on basal- and insulin-stimulated glucose transport in skeletal muscle.

The rat L6 skeletal muscle cell line used in the present study is a well-established model of skeletal muscle widely used in studies of insulin signalling and glucose transport. The L6 cells start off as myoblasts and can be induced to differentiate into myotubes (125). They express the GLUT1, GLUT3 and GLUT4 glucose transporters, the insulin receptor, IRS-1, PI3K, and Akt protein, and are

responsive to insulin (6,48,121,60). Acute insulin (100 nM) treatment causes a ~two-fold increase in glucose uptake by the cell line within 15 minutes (58). There are currently no reported studies examining the effects of naringenin on skeletal muscle glucose transport in this or other cell lines.

The present study presents for the first time findings that naringenin stimulates glucose uptake in L6 skeletal muscle cells, demonstrating that naringenin has an insulin-like effect on skeletal muscle glucose uptake. This effect of naringenin is insulin independent as the cells were not treated with insulin. The level of naringenin-mediated increase in glucose uptake is similar to that reached by insulin and the effect is time- and dose- dependent. However, the naringenin effect requires a longer incubation time to reach levels comparable to those reached by stimulation with insulin, naringenin requiring 120 minutes while insulin requiring only 30 minutes. This longer incubation time could be due to use of a different signalling cascade than that of insulin. This is plausible since other compounds (e.g. antidiabetic drug metformin) that stimulate glucose uptake appear to have different mechanisms of action than insulin (57). One possibility for using different mechanisms is differences in how compounds transmit their signal to the cell. Naringenin could either transmit a signal via interacting with molecules at the plasma membrane (e.g. receptors) or it might enter the cell and directly affect signalling molecules within the cell. Using radioactively labelled naringenin El Mohsen *et al* (21) showed that naringenin accumulates in tissues including the brain, lungs, heart, liver, spleen, and kidneys (in all tissues examined) and suggests that lack of complete recovery of the compound may be due to its accumulation in other tissues not tested such as muscle and adipose

tissue. Furthermore naringenin appears to interact with estrogen receptors which are located intracellularly (117,120). This implies that naringenin enters the cell but the exact mechanism of transport is unknown. The majority of flavonoids in their aglycone form are hydrophobic and therefore are suspected to cross the plasma membrane by simple diffusion. However in their glycosylated form they are more hydrophilic and would require transporters (62). Therefore it is likely that naringenin may utilize both passive diffusion and carrier-mediated transport as appears to be the case for the structurally similar resveratrol which has been shown to enter the cell by simple diffusion in hepatic cells and intestinal cells but may also involve carrier-mediated transport in hepatic cells (45,67). Future studies could examine transport of naringenin in skeletal muscle and the time it requires by using radioactively labelled [ $^3\text{H}$ ] naringenin as was performed for resveratrol in hepatocytes (67).

In finding that naringenin enhances basal glucose uptake it was also found that naringenin has an additive effect on sub-maximal concentrations of insulin but not on maximal insulin concentrations. This suggests that naringenin may be a useful compound for the treatment of insulin resistance and future studies could examine this possibility in models of insulin resistance. The observation that naringenin tends to increase but does not significantly change the level of glucose uptake with maximal insulin concentration could be due to naringenin treatment causing an almost maximum increase prior to addition of insulin, and therefore limiting the ability of insulin to cause any further effects on glucose uptake. It also suggests that the naringenin- and insulin-stimulated pathways may converge at some point.

The present study appears to be the only study that indicates the glucose uptake-stimulating effects of naringenin. Studies in 3T3-L1 adipocytes (40), U937 monocytes (92), and T47D and MCF-7 breast cancer cells (39) all report that naringenin inhibits basal (40,39,94) and insulin-stimulated (94) glucose uptake. Naringenin was also found to competitively inhibit glucose uptake in isolated rabbit intestinal and rat renal brush border membrane vesicles, and rat everted intestinal sleeves (71). These different effects of naringenin may be attributable to the use of different cell lines and different mechanisms of glucose transport and means of regulation employed by these cells. For instance, intestinal and renal glucose transport is largely mediated by the sodium-dependent glucose transporter, SGLT1 (71), while glucose uptake in myotubes is transported via specific isoforms of the sodium-independent GLUT glucose transporters. Other cells that respond to insulin and/or largely utilize GLUT transporters such as U937 monocytes, which express GLUT1 and GLUT3, and MCF-7 cancer cells, which express GLUT4, may still have different regulation of glucose transport and thus respond differently to the compounds. Glucose transport in adipocytes is known to be more analogous to that of myotubes and therefore the different responses could also be due to differences in methodology.

## **5.2 Elucidating the mechanism of naringenin action.**

In the present study we also report that like insulin, naringenin does not have a significant effect on glucose transport in myoblasts. Due to the properties of the L6 line of differential expression of GLUT1 and GLUT4 transporters between the myotube vs. myoblast stages (see section 3.3 in thesis) (58, 78) these results suggest

that the effect of naringenin-stimulated glucose uptake may modulate GLUT4 transporter to the plasma membrane, GLUT4 intrinsic activity, or may inhibit GLUT4 endocytosis or degradation thereby prolonging their half-life (56.1). Unlike GLUT4 which is mainly expressed in the myotube stage, GLUT1 levels are high in the myoblast stage and decline as the cells differentiate. Therefore if GLUT1 is involved one would expect an increase in basal glucose uptake in myoblasts treated with naringenin. This was not observed. Besides GLUT1 and GLUT4 the L6 also express the GLUT3 transporter which could also be involved in the naringenin-stimulated glucose uptake. This is unlikely since GLUT3 expression is similar in myoblasts as in myotubes (6,58) and so an increase in glucose uptake in naringenin-treated myoblasts would also be expected if that was the case. However this hypothesis is based on circumstantial evidence and so the effects of naringenin on the different GLUTs would need to be further examined. Some evidence of note to support evaluating modulation of GLUTs by naringenin comes from a study with the *Canna indica*, a plant traditionally used in Thai folk medicine to treat diabetes (97). An extract of this plant was found to contain several groups of flavonoids and to increase glucose uptake in L8 muscle cells, an effect that was also partially additive with insulin. Treatment with this extract increased the levels of cell surface GLUT1 and GLUT4 transporters as well as GLUT1 expression.

Future studies could look more directly at the effects of naringenin on the total and cellular localization levels of the GLUT isoforms. Total protein levels could be detected by preparing total cell lysates or total cell membranes and performing Western blot analysis using specific antibodies against GLUT1, GLUT3, and GLUT4

in the samples. The same could be done with isolated membrane fractions to examine changes in the redistribution of the GLUT isoforms intracellularly and at the plasma membranes (121). Additionally the effects of naringenin on GLUT translocation to the plasma membrane could be examined by detecting GLUT1*myc* and GLUT4*myc* cell surface levels in L6 myotubes expressing GLUT1*myc* and GLUT4*myc*, respectively. These cells express a GLUT isoform that is tagged with an exofacial *myc* epitope and the amount of GLUT1*myc* or GLUT4*myc* incorporated into the plasma membrane can be quantified immunologically (52,122). Besides the above studies it would also be important to examining the effects of naringenin on the stability of GLUT proteins at the plasma membrane and their intrinsic activity.

L6 express estrogen receptors (ER) (50) and ERs may play a role in glucose homeostasis as mice lacking the  $\alpha$  isoform of ER exhibit insulin resistance and impaired glucose tolerance (20). Recently Deng *et al* (20) presented data indicating that ERs are involved in the resveratrol-stimulated glucose uptake in muscle. Since naringenin appears to interact with ERs (117,120) it would seem valid to explore the significance of these receptors on naringenin-stimulated glucose uptake.

Our data suggests that naringenin utilizes a different mechanism from insulin to increase glucose uptake since the increase in glucose uptake did not require Akt phosphorylation/activation. Akt is a serine/threonine kinase with three isoforms: Akt1, Akt2, and Akt3. The Akt2 isoform is implicated in the involvement of insulin-stimulated glucose uptake and GLUT4 translocation (3,54,55). Full activation of Akt requires phosphorylation of two of its residues, Thr308 and Ser473 (55,114). As a result, phosphorylation of Akt at these residues has been generally accepted as an



indication of Akt activation. In our experiments naringenin treatment did not induce Akt phosphorylation at either of the residues. Previous studies with resveratrol show that increases in glucose uptake by resveratrol are also independent of Akt phosphorylation in L6 (in our lab) or C2C12 myotubes (88). Additionally, our data indicates that naringenin does not potentiate the insulin-stimulated Akt phosphorylation, indicating that its additive effect on insulin-stimulated glucose uptake is also Akt independent. This suggests that naringenin does not have insulin sensitizing properties but rather uses a different mechanism that combines with that produced by the insulin mechanism.

In this study we also examined the potential role of PI3K in mediating the effects of naringenin, a signalling molecule involved in insulin-stimulated glucose uptake. To do this we used the specific and potent PI3K inhibitor wortmannin. Wortmannin is a microbial secondary metabolite that inhibits PI3K in an irreversible and non-competitively way (96). Our results suggest that the effect of naringenin on glucose uptake is PI3K dependent since inhibition of PI3K by wortmannin significantly inhibits the naringenin-stimulated glucose uptake. In our study we tested the efficiency of wortmannin to inhibit PI3K activity indirectly by examining Akt phosphorylation on insulin-treated cells. The results indicate that wortmannin pre-treatment completely abolishes the insulin-stimulated Akt phosphorylation, implying that wortmannin successfully inhibited PI3K activity in our experiments. Future studies might directly examine the effects of naringenin on PI3K activity by performing a PI3K activity assay and measuring PIP<sub>3</sub> production. PI3K involvement has also been reported for another effect of naringenin. Borradaile *et al* showed that

naringenin has an insulin-like effect of inhibiting apolipoprotein B secretion in hepatocytes which was partially inhibited by wortmannin (9). In addition the previously mentioned increase in glucose uptake by the flavonoid-containing extract of *Canna indica* was also PI3K dependent (97). Similar results have been shown with resveratrol which increased glucose uptake in L6 myotubes in a PI3K-dependent and Akt-independent way (in our lab), and PI3K involvement was apparent in the hypoglycaemic effects of resveratrol *in vivo* (16). Interestingly resveratrol-stimulated glucose uptake in C2C12 myotubes was found to be PI3K-independent (93). This discrepancy in results for resveratrol between the two skeletal muscle models could be due to differences between the two culture models. PI3K is particularly known for modulating GLUT4 translocation and while both L6 and C2C12 myotubes express these transporters (105,58,116) the C2C12 myotubes are reported to lack the insulin-responsive vesicular compartments which appear to be necessary for proper GLUT4 trafficking (116). The observation that the same polyphenol stimulates glucose uptake in what appears to be different mechanisms in cells that are both models of skeletal muscle may suggest that the polyphenol is capable of activating more than one pathway leading to glucose uptake. It should be noted that there are also reports in the literature that both naringenin and resveratrol negatively regulate PI3K activity. In both, 3T3-L1 adipocytes and MCF-7 breast cancer cells, naringenin was found to inhibit PI3K (39,40), and resveratrol was found to inhibit PI3K-mediated glucose metabolism in b-cell lymphomas (25). One potential explanation for such a differences is tissue specific effects of the compounds.

Our finding that naringenin stimulates glucose uptake in a PI3K-dependent but Akt-independent manner is consistent with the present understanding of PI3K involvement in the insulin-stimulated glucose uptake. It is established that stimulation of the PI3K pathway by insulin is crucial for the insulin-stimulated glucose uptake. Inhibition of PI3K activity has been shown to completely abolish glucose uptake and GLUT4 translocation (15,1,55,91). However, although PI3K is required it is not sufficient for the insulin-stimulated glucose uptake. PI3K is activated by different hormones and growth factors but only activation of PI3K by some of these activators results in glucose uptake and GLUT4 translocation (31,48,1,112,113). And while in muscle both insulin and PDGF stimulate activation of PI3K and Akt phosphorylation, only insulin induces Akt phosphorylation in adipocytes (112,113). In adipocytes PI3K appears to be required for GLUT4 translocation but insulin stimulation is additionally required to render the GLUT4 transporters functional (112). These experiments suggest that there may be a PI3K-independent pathway that cross talks with PI3K to mediate glucose transport by insulin. Likewise, this may be the case with the naringenin-stimulated glucose uptake: naringenin may stimulate PI3K along with another signalling cascade, which may converge at some point. There are several isoforms of PI3K and because we did not observe Akt stimulation despite PI3K involvement it is possible that naringenin's action depends on a PI3K isoform that is not involved in the stimulation of Akt.

An alternative way of stimulating glucose uptake in skeletal muscle is via activations of AMPK, though the signalling pathway is still elusive and seems varied depending on the stimulus. We report that naringenin increases AMPK

phosphorylation in skeletal muscle cells. AMPK activation has also been implicated in mediating the antidiabetic effects of exercise, metformin, berberine, and resveratrol (13,28,84,35,126,110,119). AMPK is a protein kinase composed of three subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$  (28,118). Activation of AMPK requires phosphorylation at its Thr172 residue (35,43,71) and this has been acknowledged as indicating activation. In mammalian cells AMPK acts as a sensor of change in cellular energy status, regulates numerous processes of cellular metabolism, integrates nutritional and hormonal signals in the peripheral tissues and the hypothalamus and mediates the effects of adipokines in regulating food intake, body weight and glucose and lipid homeostasis. With such varied metabolic effects it is not surprising that AMPK would have numerous ways of being activated. Activators of AMPK include an increase in AMP:ATP ratio (13,35,37,56), and its upstream kinases LKB1 (32, 35) and CaMKK (35).

PI3K has also been implicated in the activation of AMPK. Zou *et al* (126) found that activation of AMPK by metformin in cultured bovine aortic endothelial cells was PI3K dependent. They showed that pharmacological inhibition of PI3K by wortmannin or LY294002 inhibited both AMPK phosphorylation and activity caused by metformin but did not inhibit AICAR-induced AMPK activity. In our study inhibition of PI3K by wortmannin did not inhibit the naringenin-stimulated AMPK phosphorylation. As mentioned earlier we confirmed the potency of wortmannin as a PI3K inhibitor by testing its effectiveness on insulin-stimulated Akt phosphorylation and observing that this effect was abolished. This indicates that PI3K is not the mediator of naringenin-induced AMPK phosphorylation in L6 as it would seem based on the study of Zou *et al* with aortic endothelial cells. Our finding does not rule out

the possibility that AMPK and PI3K converge on some other point downstream of AMPK.

Recently, research by Hou *et al* (46) showed that SIRT1 is activated by polyphenols, which included resveratrol and a synthetic polyphenol, and mediates their effect to increase LKB1 phosphorylation and AMPK activity in hepatocytes. They showed that inhibition of SIRT1 pharmacologically and genetically abolished the stimulating effects of polyphenols on AMPK. SIRT1 has also been shown to deacetylate LKB1 in HEK293T cells, which was associated with an increase in LKB1 activity and AMPK phosphorylation. Down-regulation of SIRT1 on the other hand reduced phosphorylation of AMPK (66). SIRT1 is a member of the sirtuin family of NAD<sup>+</sup>-dependent protein deacetylases that are implicated to be involved in the beneficial biological effects of caloric restriction (CR) (47). One of the benefits of CR in animals appears to be a slight reduction in blood glucose levels and relative resistance to diabetes and resveratrol and other polyphenols appear to be CR mimetics (106). Resveratrol has been shown in an *in vitro* assay to directly stimulate SIRT1 activity by ~13 fold compared to control, and several other polyphenols tested, including naringenin, also directly stimulated SIRT1 activity, albeit only ~2-fold for naringenin (47). Based on the evidence that SIRT1 appears to regulate AMPK activity including that induced by resveratrol as well as that polyphenols activate SIRT1 we hypothesized that SIRT1 might be involved in naringenin-stimulated glucose uptake via AMPK activation. In our study SIRT1 inhibition by either nicotinamide or Ex527 does not reduce naringenin-stimulated glucose uptake, AMPK phosphorylation or LKB1 phosphorylation suggesting that SIRT1 does not play a role

in these effects of naringenin. It is possible that the role of SIRT1 on AMPK activation is tissue specific since one study reported that in skeletal myoblasts AMPK is upstream of SIRT1 (30). Our data also suggests that the observed AMPK phosphorylation does not seem to be mediated by LKB1, since its phosphorylation was not increased by naringenin. Phosphorylation of LKB1 occurs after it is activated and so it can be used as an indication of LKB1 activation. However, phosphorylation is not required for activation of LKB1 and so it weakens the validity of LKB1 phosphorylation as an indicator of LKB1 action (66).

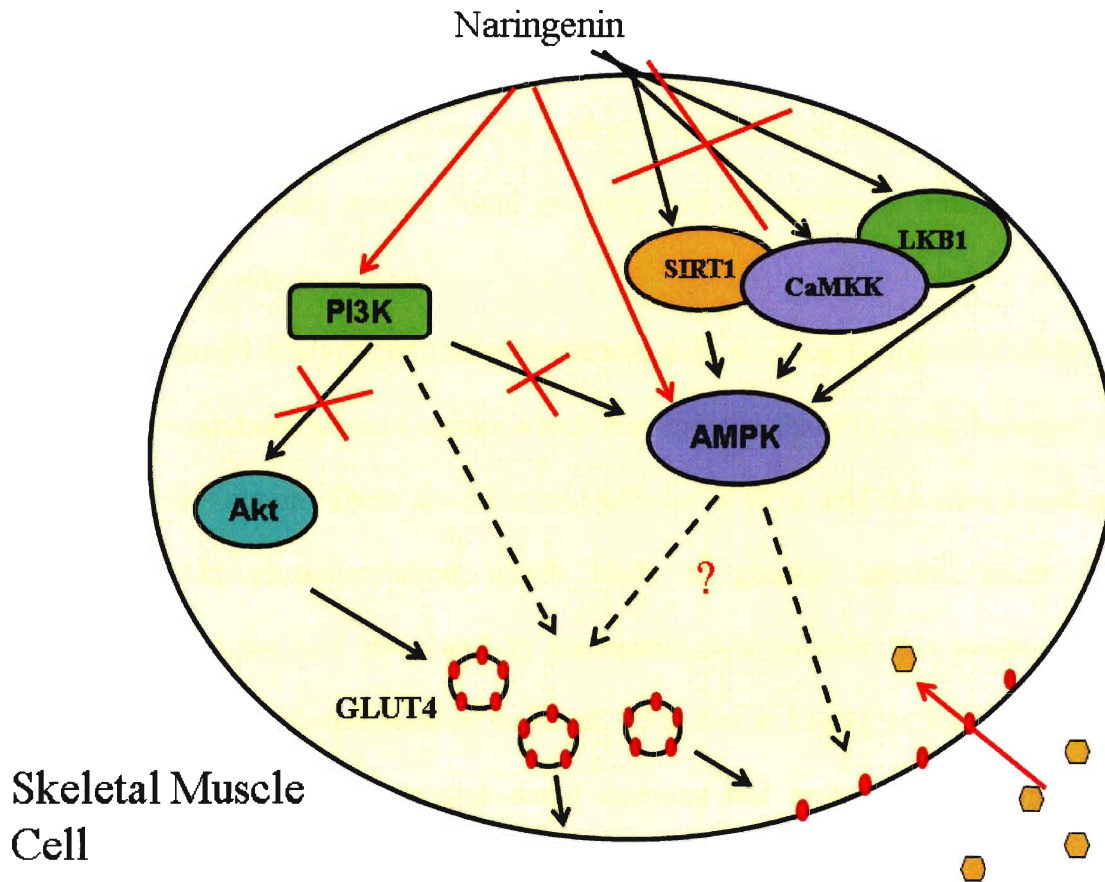
AMPK can also be activated by CaMKK. Phosphorylation of Thr172 by this kinase is stimulated by a rise in calcium rather than an increasing AMP: ATP ratio (35). Although muscle-specific knockouts of LKB1 suggest that the calcium-mediated pathway does not have a significant contribution in skeletal muscle, the contraction-induced glucose transport in skeletal muscle cells is believed to be in part mediated by increased cytosolic calcium levels and activation of the CaMKK-dependent AMPK, as is suggested to be the case for mouse skeletal muscle during mild tetanic contractions (35,49). It has also been shown that oxytocin-stimulated glucose uptake in C2C12 cells uses a mechanism that involves activation of CaMKK and AMPK (69). Therefore this kinase may be a mediator of AMPK activation in muscle by some stimuli and its involvement in naringenin-stimulated glucose uptake was examined. We found that inhibition of CaMKK by STO-609 does not inhibit naringenin-stimulated glucose uptake, indicating that this kinase is not involved in this action of naringenin. The effects of STO-609 on naringenin-stimulated AMPK phosphorylation was not examined by us so it is possible that CaMKK mediates

naringenin-stimulated AMPK phosphorylation but that this stimulation of AMPK is not involved in naringenin-stimulated glucose uptake. Therefore future studies could examine whether inhibition of CaMKK abolishes AMPK phosphorylation induced by naringenin. Additionally, measurements of intracellular calcium would shed light on whether naringenin induces high intracellular calcium level, which could activate CaMKK.

Another way of activating AMPK is by an increase in AMP:ATP ratio (13,35,37,56). Therefore we examined whether inhibition of AMPK by compound C (CC) would have an impact on naringenin-stimulated glucose uptake. Our results indicate that compound C does not have an effect on naringenin-stimulated glucose uptake. Thus far we do not have evidence suggesting that AMPK mediates the naringenin-stimulated glucose uptake and although the results seem to suggest against it the possibility cannot be ruled out. CC is an ATP-competitive AMPK inhibitor. Since AMP and ATP competitively bind to the same site on AMPK (51), the effect of CC in the cell would seem to decrease the chance of AMP binding to the AMPK site thereby mimicking a state of decreased AMP:ATP ratio. The mechanism of CC makes it an imperfect AMPK inhibitor since AMPK is activated by a variety of stimuli some of which do not alter the AMP:ATP ratio (13,126,111). Besides the aforementioned CaMKK (35), metformin is another example reported to stimulate AMPK phosphorylation without requiring an increase in AMP:ATP (126). Metformin acts as an inhibitor of mitochondrial complex I of the respiratory chain and therefore may cause a decrease in ATP production. However, some studies report that the activation of AMPK by metformin occurs without affecting the AMP:ATP ratio and

Zou *et al* found that  $\text{ONOO}^-$  activates AMPK without changes in cellular ATP or AMP levels (126). In light of this information it was important to determine whether CC was an effective inhibitor of AMPK in the case of naringenin. Our data suggests that CC is effective in inhibiting naringenin-stimulated AMPK phosphorylation. These results suggest that naringenin stimulates AMPK phosphorylation but this effect is not necessarily required for naringenin to stimulate glucose uptake. Future studies could use small interference RNA (siRNA) to silence AMPK protein expression to examine with more clarity if AMPK has a role in naringenin-stimulated glucose uptake. This approach has been used by Konrad *et al* (61) to examine the mechanism of antidiabetic drug troglitazone on glucose transport in the L6 line. Alternatively a dominant-negative (DN) AMPK could be used that has a mutation in the  $\alpha$ -subunit resulting in a kinase dead form of AMPK as was performed by Mu *et al* (81).





**Figure 17:** Schematic of results summary. Naringenin stimulates glucose uptake in L6 myotubes. The effects of naringenin are PI3K-dependent but Akt-independent. Naringenin stimulates AMPK phosphorylation. The effects of naringenin on glucose uptake and AMPK stimulation are SIRT1 independent. The naringenin-stimulated glucose uptake is not inhibited by CaMKK or AMPK inhibition. Activation of AMPK by naringenin may not be necessary for its stimulatory effect on glucose uptake.

### 5.3 Limitations and Future Directions

Future studies could utilize siRNA to inhibit AMPK expression in L6 skeletal muscle or DN AMPK to determine more conclusively the AMPK molecule's role in naringenin-mediated glucose uptake. The effect of naringenin on GLUT1, GLUT3, and GLUT4 levels and activity could be examined. This study focused on the *in vitro*,

acute effects of naringenin in L6 skeletal muscle cells but further research could extend the study of naringenin to examine chronic effects of naringenin, effects on other insulin target cells and *in vivo*, as well as *in vitro* and *in vivo* models of insulin resistance. Also, future studies could examine how naringenin is transported into skeletal muscle cells.

The preset study found that pretreatment with wortmannin partially inhibited naringenin-stimulated glucose uptake which may indicate that PI3K may be involved in naringenin's action. There are different isoforms of PI3K and the class I isoform stimulates Akt phosphorylation which leads to glucose uptake. Since Akt phosphorylation was not stimulated by naringenin it is possible that another PI3K isoform may be involved. Another target of PI3K that is known to lead to glucose uptake is PKC and future studies could examine this protein's involvement in naringenin-stimulated glucose uptake. A major limitation of the present study is that wortmannin at a 1  $\mu$ M was used to examine the involvement of PI3K. This concentration of wortmannin has been used previously by others in skeletal muscle culture including the L6 cell line (119.1, 124.1). However, at this concentration wortmannin is not very specific and not only inhibits all PI3K isoforms, which all have an IC<sub>50</sub> values in the nm range, but also several other proteins including mTOR, DNA-dependent protein kinase, and myosin light chain kinase (16.1, 60.1). Therefore it cannot be concluded that PI3K is involved, and studies directly examining PI3K activity would be beneficial to determine whether naringenin increases PI3K activity.

A limitation in the present study is that glucose uptake was assessed only using 2-[<sup>3</sup>H]deoxy-D-glucose (2DG) while 3-O-[methyl-<sup>3</sup>H]-D-glucose was not used.

Once inside a cell 2DG can be phosphorylated by hexokinase although it cannot be further metabolized in glycolysis. Because of this, hexokinase activity can influence the rate of 2DG uptake by altering the concentration gradient for 2DG entry and so changes in glucose uptake measurements could be due to changes in glucose facilitated transport and/or glucose phosphorylation rate. In the present study the observed changes in glucose uptake levels should indicate changes in facilitated transport since the L6 cells were incubated with 2DG for 10 minutes and it has been reported by others (56.2) that for the first 10 minutes 2DG uptake into L6 cells is limited by transport. Nevertheless, the possibility exist that 2DG uptake levels are not only determined by glucose transport. The use of 3-*O*-[methyl-<sup>3</sup>H]-D-glucose, which does not get phosphorylated, would be useful to confirm that the naringenin-stimulated glucose uptake observed in the study is due to increased 2DG transport rather than increased phosphorylation of 2DG (56.2).

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## APPENDIX I:

The results in this thesis, that naringenin stimulates glucose uptake in L6 skeletal muscle, are in contrast with several studies that indicate that naringenin inhibits glucose uptake. As mentioned earlier in the discussion section of the thesis, these contrasting results could be due to tissue/cell type specific effects or to differences in methodology. Upon examination of the method sections of these studies reporting inhibitory effects of naringenin I observed that all three follow a protocol of including the test compounds in a buffer to which the radioactively labeled glucose is then added. In the glucose uptake assays I followed a protocol in which the test compounds were removed by washing before the uptake assay was performed. Some of my last experiments examined the effects of incubating naringenin in the glucose uptake buffer on glucose transport. This preliminary data is presented here. The results indicate that when myotubes are treated with naringenin and prior to performing the glucose uptake assay the media containing naringenin is removed, the result is an increase in glucose uptake. However, if the glucose uptake assay is performed in the presence of naringenin, the result is a prevention of the increase in glucose uptake of naringenin-treated cells originally observed when naringenin was excluded from the uptake buffer. This inhibition is so great that it not only abolishes the increase in glucose uptake but also inhibits it below basal glucose uptake. Furthermore, this inhibition is not due to an osmotic effect since neither exposure to sucrose or mannitol in the same concentration as naringenin affected basal or naringenin-stimulated glucose uptake (see Figure and details at end of

Appendix I). These preliminary results suggest that naringenin may be an inhibitor of glucose uptake and further research needs to be performed to explore this possibility.

Besides naringenin, similar contrasting reports are present for the effects of other flavonoids and resveratrol on glucose uptake *in vitro*. On one hand polyphenols are reported to stimulate glucose uptake in several cell lines, on the other, many of the same polyphenols are reported to inhibit glucose uptake. Resveratrol has been shown to stimulate glucose uptake in two skeletal muscle culture models, L6 and C2C12 (11,93); to have no significant effect on glucose uptake in human diffuse large B-cell lymphomas (25); and to inhibit glucose uptake in human transformed myelocytic cells (U937 and HL-60) (94) and ovarian cancer cells (64). The polyphenolic/flavonoid containing extract from *Canna indica* has been reported to increase glucose uptake, GLUT1 and GLUT4 content at the plasma membrane, and GLUT1 expression in L8 muscle cells (97). Also, the flavonoids kaempferol and quercetin are reported to increase insulin-stimulated glucose uptake in 3T3-L1 cells (26). On the other hand, there are numerous reports indicating that flavonoids are inhibitors of glucose transport. Flavonoids are reported to inhibit glucose uptake in porcine jejunum brush-border membrane vesicles (quercetin monoglucosides) (15), isolated rat adipocytes (myricetin, quercetin, catechin-gallate) (109), human erythrocytes (numerous flavonoids) (76), *Xenopus laevis* oocytes (numerous flavonoids) (62), and MC3T3-G2/PA6 adipose cells (numerous flavonoids) (89). Interestingly quercetin is reported to have varied effects between a model of adipocytes and isolated rat adipocytes (26, 62).



The methods sections of these reports consistently exhibit the protocol difference in the glucose uptake assays between the studies that indicated stimulatory vs. inhibitory effects of polyphenols, as was shown for naringenin. Among the studies where similar methods were used the studies that showed a stimulatory effect followed a protocol in which the test compounds were removed by washing before the uptake assay was performed (11,26,97). On the other hand, the studies reporting inhibitory effects followed a protocol which included the test compounds in a buffer to which the radioactively labelled glucose was then added (40,39,94,71,95,62,89). A few of the studies did not have sufficient information to determine which protocol was used (93,64). A major difference between the protocols is the removal of the potential inhibitor before performing glucose uptake measurements, thus removing the inhibitory stimuli.

Several of the studies that indicate inhibitory effects on glucose transport suggest that the nature of the inhibition is via inhibition of the glucose transporters and not necessarily only through regulation. In the myelocytic cells the inhibition by resveratrol was competitive and also inhibited dehydroascorbic acid uptake, which is known to occur via GLUT1 and GLUT3 isoforms (94). To elucidate whether the means of inhibition of glucose and dehydroascorbic acid uptake is by blocking the GLUT transporters the effects were also tested in Chinese hamster ovary (CHO) cells overexpressing GLUT1 or GLUT3. Overexpression of either of these isoforms increased glucose and dehydroascorbic acid uptake and resveratrol dose-dependently inhibited both of their uptakes (94). Quercetin monoglucosides were also shown to competitively inhibit SGLT1 glucose uptake in porcine jejunum brush-border

membrane vesicles (15). Some studies have looked specifically at the effects of flavonoids on glucose transporters in order to identify the means by which flavonoid glucosides are transported into cells (15). Unlike their aglycone forms, which are hydrophobic, flavonoid glycosides are hydrophilic and need transporters to cross the plasma membrane. The glucose transporters were considered candidates for this action. In this respect Kottra and Daniel (62) examined the effects of flavonoids with and without attached glucose residues on the sodium-dependent SGLT1 transporters in *Xenopus laevis* oocytes. By testing transport currents generated by different flavonoids in *Xenopus laevis* oocytes expressing SGLT1 they showed that none of the flavonoids tested, including naringenin, are substrates for SGLT1 nor are they transported through it. However, the flavonoid glycosides as well as some of the aglycones were effective competitive inhibitors of SGLT1 and had high affinity for the transporter, the degree of which was structurally dependent. The aglycone form of naringenin was a non-competitive potent inhibitor, while its glycosylated form was competitive. The researchers speculated that flavonoids can bind the transporter with either its sugar moiety or another part of the molecule (62).

Evidence also exists for flavonoids interacting with some isoform of the GLUT transporter family. In *Xenopus laevis* oocytes expressing the GLUT2 transporter different classes of flavonoids were found to inhibit GLUT2-mediated glucose transport (from 62). Several flavonoids have also been shown to inhibit GLUT1-mediated glucose uptake through what appears to be a direct interaction with it (109). The study by Strobel *et al* (109) in isolated rat adipocytes showed that several flavonoids competitively inhibited glucose uptake. As one part of their study

they developed a three-dimensional molecular comparative model of the GLUT4 transporter in order to examine molecular dynamics of flavonoid interaction with GLUT4. Their simulation with flavonoids revealed a direct, non-bonded interaction with the transporter, as was found for D-glucose (109).

If polyphenols are in fact interacting with glucose transporters in a way that inhibits glucose transport, why the contrasting results indicating that flavonoids increase glucose uptake? An interesting hypothesis presents itself. If certain polyphenols bind to glucose transporters and potentially inhibit their ability to transport glucose then this action could mimic a state of glucose deprivation for the cells. Glucose is known to regulate its own transport and a state of glucose deprivation is known to have stimulatory effects on glucose transport in muscle cells, cultured fibroblasts, cardiocytes, adipocytes, and glial cells (121, 57, 60). In L6 muscle cells chronic and acute glucose deprivation was shown to stimulate glucose uptake above basal, resulting in a 4-fold increase in glucose uptake within 2 hours that remained elevated for over 22-hours (121). The results suggest that glucose deprivation causes GLUT1 translocation to the plasma membrane and activation of its intrinsic activity, followed by an increase in the rate of GLUT1 transporter mRNA transcription and protein synthesis in the chronic glucose deprivation state (121). In another study glucose deprivation was also shown to increase GLUT4 levels in the plasma membrane (60). In this respect an important weakness of my preliminary data is that the glucose uptake assay was not performed under *in vivo*-like conditions. The uptake assay is performed with only 10  $\mu$ M 2-deoxy-D glucose while prior to, when the cells are incubated with naringenin for two hours, the culture media contains 5 mM of

glucose to mimic an *in vivo* condition. Therefore under these conditions the relative low quantity of naringenin to glucose may not adequately inhibit glucose uptake to induce a glucose deprived-like state. This concern points to the preliminary nature of the present results and the need to perform experiments with naringenin in various glucose concentrations to determine the effect on  $K_m$  and  $V_{max}$  of glucose uptake and the potency of naringenin as an inhibitor.

One explanation of why glucose restriction (or glucose transporter inhibition) leads to improved glucose uptake may be that it creates a mild stress on the cells which causes them to induce a survival response. This is consistent with the Hormesis hypothesis that aims to explain the health benefits of caloric restriction (CR) (106). This hypothesis states that CR confers health benefits by creating a mildly stressful condition within an organism that stimulates a survival response which alters metabolism and activates defence mechanisms against aging (106, 47). Notably, one of the health benefits of caloric/glucose restriction has been on type II diabetes by improving insulin sensitivity and maintaining reduced blood glucose levels. Animals on caloric restriction show lower blood glucose levels and appear to be relatively resistant to the development of diabetes (106). This appears to be due to an increase in glucose uptake in skeletal muscle and fat pads resulting from an increase in GLUT4 at the plasma membrane (106). The Hormesis hypothesis of CR is also the bases for understanding why polyphenols mimic the beneficial effects of CR. Polyphenols, which are produced by plants under stress, have been shown to activate stress-response pathways in yeast, worms and flies, and it is suspected that these and other organisms have evolved to recognize these molecules as stress signals

indicative of an unfavourable environment and adopt changes to enhance their survival (106). Thus it is suspected that polyphenols may stimulate the same inherent defence mechanisms as CR. This was suggested to be the case for resveratrol in a study by Howitz *et al* (47) who showed that resveratrol extended lifespan of *Saccharomyces cerevisiae*, as was reported for caloric restriction, but glucose restriction did not result in extension of lifespan of resveratrol-treated cells (47). Furthermore, there are several signalling molecules including SIRT1 and AMPK, that are reported to be modulated by both polyphenols and CR (30, 88).

In summary, the literature indicates that polyphenols, specifically different subclasses of flavonoids and resveratrol, possess both stimulatory and inhibitory effects on glucose transport. This could be due to tissue specific effects but there also appears to be a slight but consistent difference in the protocol used in the glucose uptake assay. The significance of this difference needs to be further examined, particularly in an *in vivo*-like glucose state. In some cases the inhibitory effects of flavonoids appear to be due to their interaction with and blockade of the glucose transporters, which gives credence to the importance of the difference in the protocol (i.e. inclusion vs. exclusion of the compounds in the uptake buffer). Although seemingly contradictory, initial inhibition of glucose uptake by these compounds may not necessarily be counterintuitive since glucose deprivation stimulates glucose uptake in a variety of cell types. The possibility that certain polyphenols may create a state of glucose restriction and in turn activate a counter-acting stress response seems feasible since mild stress induction, which is the bases of the Hormesis hypothesis, explains the health benefits of caloric restriction, and because some polyphenols are

considered mimetics of caloric restriction, as evidenced by their ability to mimic the same responses and modulate some of the same signalling molecules as caloric restriction.

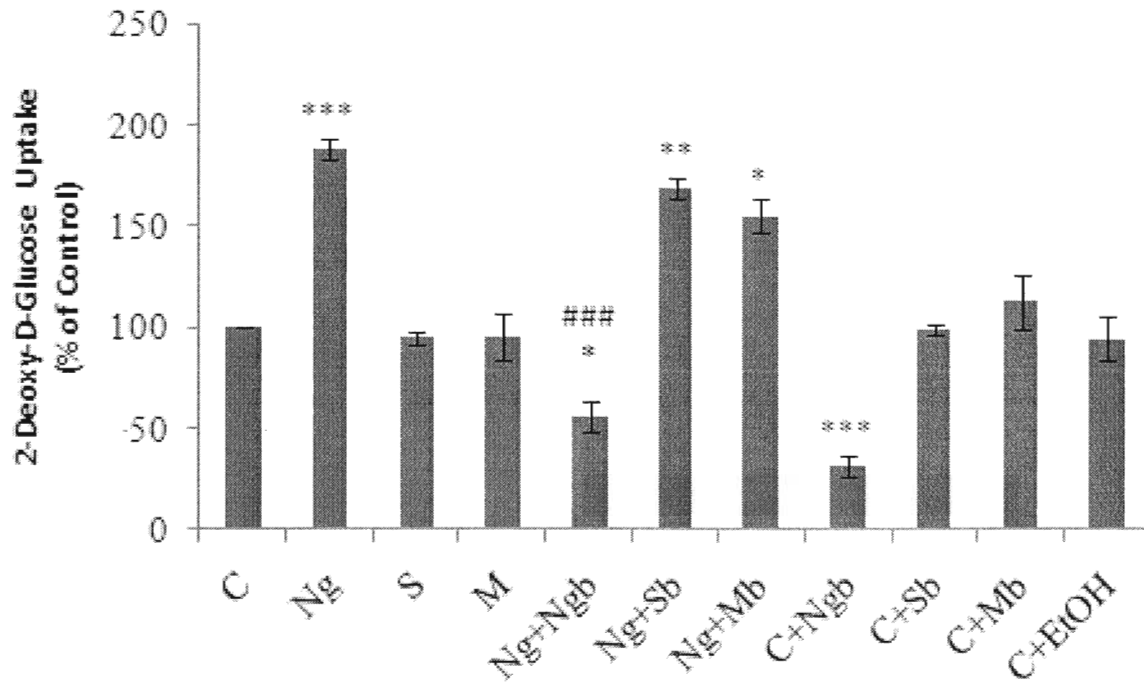
### **Effects of naringenin incubation with glucose uptake buffer on glucose uptake.**

In following the glucose uptake protocol used in our lab we remove the media with our test compounds then wash out any residue before incubating with the radioactively labeled glucose uptake buffer. Because the uptake buffer does not contain the test compounds in it, in this case naringenin, it is important to examine what direct effects naringenin would have on glucose transport. To tests this myotubes were treated with or without 100  $\mu$ M naringenin for 2 hours. At end of treatments [ $^3$ H] 2-deoxy-D glucose uptake was performed but some cells were incubated with regular glucose uptake buffer while others were incubated with the glucose uptake buffer supplemented with 100  $\mu$ M naringenin. There was also a possibility that any differences in glucose uptake could be due to either a specific action of naringenin or to an osmotic effect created by naringenin. Therefore to control for the osmotic effect some groups of cells were incubated with an uptake buffer supplemented with 100  $\mu$ M of sucrose or mannitol. Mannitol is a sugar alcohol used to control for osmolarity. Sucrose was used since as a disaccharide it is not absorbed by cells which do not have transporters to aid across the plasma membrane. One group of cells was also incubated with glucose uptake buffer supplemented with the vehicle (0.1% ethanol).

As before, treatment with 100  $\mu$ M naringenin for 2 hours followed by incubation in regular buffer led to a significant increase in glucose uptake ( $189 \pm 5.2\%$

of control,  $p < 0.001$ ). However, treatment with naringenin followed by incubation with naringenin-supplemented uptake buffer led to abolishment of glucose uptake originally observed which was also significantly below basal levels ( $56 \pm 7.4\%$  of control,  $p < 0.001$  and  $p < 0.05$ , respectively). Naringenin-supplemented uptake buffer significantly inhibited basal glucose uptake of untreated cells ( $32 \pm 4.9\%$  of control,  $p < 0.001$ ). Treatment with  $100 \mu\text{M}$  sucrose or mannitol for 2 hours followed by incubation with normal uptake buffer had no significant effect on glucose uptake ( $95 \pm 3.4\%$  and  $95 \pm 11.8\%$  of control), nor did the sucrose- or mannitol-supplemented uptake buffer have an effect on naringenin-stimulated increase in glucose uptake or basal levels ( $169 \pm 4.9\%$ ,  $155 \pm 8.4\%$ ,  $99 \pm 2.3\%$ , and  $113 \pm 13.3\%$  of control, respectively). These results suggest that naringenin has a specific inhibitory effect on glucose transport into myotubes.

It is interesting to consider that differences in technique of [ $^3\text{H}$ ] 2-deoxy-D glucose uptake measurements might explain differences in findings that naringenin, or any other compound for that matter, is an inhibitor of glucose uptake in some studies and an activator in others as it was here. As an aside it was interesting to examine if the same effect would be observed with resveratrol and our finding suggest that resveratrol-supplemented uptake buffer also has an inhibitory effect on glucose uptake.



Effects of naringenin in uptake buffer on glucose uptake. L6 myotubes were serum deprived and pre-treated with or without 100  $\mu$ M naringenin, sucrose, or mannitol for 2 h. At end of treatments [ $^3$ H] 2-deoxy-D glucose uptake was performed with either normal uptake buffer, 100  $\mu$ M naringenin-supplemented uptake buffer, 100  $\mu$ M sucrose-supplemented uptake buffer, 100  $\mu$ M mannitol-supplemented uptake buffer, or 0.1% ethanol supplemented uptake buffer. Results are the mean  $\pm$  SE of 3-4 experiments expressed as percent of control. \* $p$  < 0.05, \*\*  $p$  < 0.01, \*\*\* $p$  < 0.001 compared to untreated control. ### $p$  < 0.001 compared to naringenin treated and incubated in normal buffer. C = control, Ng = naringenin treated, S = sucrose treated, M = mannitol treated, Ngb = naringenin-supplemented uptake buffer, Sb = sucrose-supplemented uptake buffer, Mb = mannitol-supplemented uptake buffer, EtOH = ethanol-supplemented uptake buffer.